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13. ABSTRACT *(Maximum 200 Words)*

The aim of this proposal is to develop a method to target novel cytotoxic agents specifically to sites of metastatic prostate cancer. In the original proposal, proteasome inhibitors were selected as the cytotoxic agent. Initial studies revealed that this approach was not tenable due to the inherent instability of these compounds. An alternative agent was then selected to continue this targeted prodrug approach. Thapsigargin (TG) induces apoptosis in a proliferation independent manner in prostate cancer cells. This cytotoxicity, however, is not prostate cell type specific and TG could not be given systemically without significant toxicity. To achieve targeted cytotoxicity the TG analogs will be converted to inactive prodrugs by coupling to a peptide carrier that is a substrate for the serine protease activity of Prostate-Specific Antigen (PSA). Since PSA is expressed in high levels only by normal and malignant prostate cells, this approach should allow specific targeting of the killing ability of TG to prostate cancer cells. Therefore a series of amine containing TG analogs have been synthesized and characterized for their ability to induce apoptosis in prostate cancer cell lines. The lead TG analog has been chemically linked via a peptide bond to a previously identified PSA-specific peptide (i.e. 6 amino acids) to produce an inactive prodrug. This prodrug can be hydrolyzed by PSA and a 25-fold increase in toxicity is seen in the presence of enzymatically active PSA. In vivo studies using this lead TG prodrug to treat PSA-producing human prostate cancers are underway. Additional prodrugs will also be synthesized in order to optimize PSA-targeting.

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Apoptosis, Drug Delivery System, Thapsigargin, Prostate-Specific Antigen (PSA), Prodrug

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INTRODUCTION:

Approximately forty thousand American men die annually from metastatic prostate cancer. Standard chemotherapeutic agents have been ineffective at significantly prolonging the survival of men with metastatic prostate cancer and these agents are typically associated with often severe, dose-limiting side effects. New agents are therefore urgently needed. While a large number of cytotoxic agents have been demonstrated to be effective in vitro, these agents are typically general cytotoxins that cannot be administered to patients without severe systemic toxicities. Therefore, what is required is a method to target the delivery of novel, effective cytotoxic agents specifically to sites of metastatic prostate cancer. Such an approach would result in increased concentration of drug within the tumor while avoiding significant systemic toxicity. One such novel agent that has been demonstrated in recent studies to induce apoptosis in a variety of cell types, including prostate cancers, is the natural product thapsigargin (TG). TG potently inhibits the Endoplasmic Reticulum Ca^{2+}-ATPase pump causing a sustained elevation of intracellular calcium that leads to induction of apoptosis in TG-treated cells. The cytotoxicity of TG, however, is not prostate cancer specific. In this proposal, a prostate cancer specific targeting strategy is outlined that will overcome this limitation. To achieve targeted cytotoxicity a potent TG analog will be converted to an inactive prodrug by coupling to a peptide carrier such that the analog can be efficiently converted back to an active killing agent only upon proteolysis by the serine protease activity of a unique prostate-specific protein, Prostate-Specific Antigen (PSA). Since PSA is expressed in high levels only by normal and malignant prostate cells and not in any significant amounts by other normal cell types, this approach should allow specific targeting of the killing ability of TG to prostate cancer cells. Therefore a series of primary amine containing TG analogs will be synthesized and characterized for their ability to induce apoptosis in prostate cancer cell lines and normal fibroblasts. Cytotoxic primary amine containing TG analogs will be chemically linked via a peptide bond to a previously identified PSA-specific peptide (i.e. 6 amino acids) to produce inactive prodrugs. Prodrugs in which the active TG analog can be efficiently released by the proteolytic activity of PSA will be tested for their potency and selectivity as PSA activated killing agents against PSA-producing, androgen independent human prostate cancer cells. The lead prodrug (i.e. the prodrug most efficiently and specifically hydrolyzed by PSA to release most active TG analog) will then be tested in vivo for activity in mice bearing PSA-producing human prostate cancers. These studies will serve to identify the best candidate prodrug that will subsequently tested in clinical trials as treatment for metastatic prostate cancer.

BODY:

The hypothesis of this proposal was that the proteolytic activity of PSA, which is highly expressed by androgen independent prostate cancer cells, can be used to activate prodrugs specifically to cytotoxic metabolites at sites of metastatic prostate cancer. Originally, the specific cytotoxic agents to be targeted were proteasome inhibitors. The original plan of the Award proposal was to develop prodrugs consisting of a proteasome inhibitor coupled to a peptide. The peptide was designed to be a specific substrate for the proteolytic activity of prostate-specific antigen (PSA). In this approach the inactive proteasome inhibitor/peptide prodrug could be given systemically without significant toxicity because PSA is enzymatically active within the blood due to complex formation with serum protease inhibitors. The extracellular fluid of prostate cancers contains large amounts (i.e. mg/ml) of enzymatically active PSA capable of releasing the active drug and inducing apoptosis of the surrounding cells.

The first two tasks of the original proposal were as follows:

Task 1: Synthesis of amine containing proteasome inhibitors and characterization of proteasome inhibition and cytotoxicity (1-12 months).

Task 2: Prodrug synthesis and determination of rates of PSA proteolysis of proteasome inhibitor prodrugs (12-24 months).

For task 1, several amine containing proteasome inhibitors were synthesized (table 1) in collaboration with chemists at Cephalon, Inc. For these inhibitors a primary amine was incorporated into the structure of the inhibitor to allow for coupling to a peptide. These inhibitors were characterized for their ability to inhibit the proteasome in a broken cell proteasome assay, table 1. These compounds demonstrated potent inhibition of the proteasome at low nanomolar concentrations. In whole cell cytotoxicity assays, these compounds were far less potent with IC_{50} values for inhibition of cell growth in the micromolar range (i.e. 1000-fold less potent), table 1.

For task 2, the proteasome inhibitor 6481 (table 1) was coupled to the PSA-specific peptide carrier Acetyl-His-Ser-Ser-Lys-Leu-Gln (Ac-HSSKLQ). This produced a compound that was still capable of potently inhibiting the proteasome in broken cell preparations with an IC_{50} value of 3 nM but demonstrated no cellular cytotoxicity at doses up to 50 μM. This prodrug was then incubated with enzymatically active PSA and assayed for hydrolysis by HPLC analysis. No demonstrable hydrolysis of the prodrug was observed (data not shown). In addition, HPLC analysis demonstrated that this proteasome inhibitor prodrug compound was unstable and rapidly degraded. The possible mechanism for this degradation is a reaction between the epsilon-primary amine of lysine with the boronic ester active group in the proteasome inhibitor. HPLC analysis also demonstrated instability of the uncoupled proteasome inhibitor 6481 by a potentially similar mechanism. Alternatively, the boronic ester group may not be stable in aqueous environment.

Originally, Cephalon Inc. had agreed to supply my laboratory with a series of modified proteasome inhibitors. This company also agreed to help with the synthesis of proteasome inhibitor-peptide prodrugs. However, on the basis of these preliminary unfavorable results, Cephalon Inc. did not wish to continue collaboration on this project. The company sited financial constraints and made a decision not to put any more of their already limited funds into the proteasome project.

These preliminary data obtained from work outlined in task 1 and 2 of the original application suggest that these proteasome inhibitors may not be ideal candidates for coupling to a peptide carrier due to their inherent instability and poor cell penetration. Instead of abandoning the project at this point, I chose to continue the work using an alternative cytotoxic agent. This decision was based on the previous findings in my laboratory suggesting that the defined PSA-specific peptide carrier could be used to effectively target a chemotherapeutic agent to sites of PSA-producing prostate cancer. In this original work, a doxorubicin analog was coupled to the HSSKLQ peptide carrier to produce a prodrug that was stable and inactive in the absence of enzymatically active PSA (Appendix 1). However, in the presence of active PSA, the cytotoxic doxorubicin analog is released and cells underwent apoptosis. These preliminary studies with the doxorubicin prodrug have provided the rationale for further development of this PSA-based targeting strategy. In recently published work sponsored by this award, a PSA-doxorubicin prodrug was tested in vivo against a PSA-producing human prostate cancer xenograft (Appendix 2) . This prodrug was found to

be non-toxic to the treated animals and significantly inhibited the rate of tumor growth. Doxorubicin has been tested previously as treatment for metastatic prostate cancer and, although partial responses were seen in some studies, this agent was not thought to be very effective therapy. Therefore, although doxorubicin may not be the preferred agent, other highly potent, novel cytotoxic agents could be employed in a similar PSA-targeted approach.

The original hypothesis of the proposal was that the proteolytic activity of PSA, which is highly expressed by androgen independent prostate cancer cells, could be used to activate prodrugs specifically to liberate a cytotoxic agent at sites of metastatic prostate cancer. An example of one such cytotoxic agent is the natural plant product thapsigargin (TG) that has been demonstrated in recent studies to induce apoptosis in a variety of cell types, including prostate cancers, figure 1. TG has the unique ability to induce apoptosis in a proliferation independent manner. Therefore, it may be an ideal agent to treat slowly proliferating metastatic prostate cancer. TG would be difficult to administer systemically because its cytotoxicity is not prostate cancer specific and it is also able to kill G_0 arrested cells To achieve targeted cytotoxicity, TG analogs can also be converted to inactive prodrugs by coupling to a peptide carrier such that they can only be efficiently converted back to active killing agents only upon proteolysis by PSA.

Thapsigargin Background

Thapsigargin (TG) is a sesquiterpene g-lactone isolated from the root of the umbelliferous plant, *Thapsia garganica*. TG has been shown to increase intracellular Ca^{2+} and induce programmed cell death in prostate cancer cell lines as well as a host of other normal and malignant cell types. More recent studies have shown that TG inhibits the sarcoplasmic/endoplasmic reticulum (ER) Ca^{2+}-ATPase (SERCA) pump with an IC_{50} value of 30 nM. This inhibition is not only efficient but also highly specific, since neither plasma membrane nor red blood cell Ca^{2+}-ATPase is inhibited, even at micromolar concentrations of TG. Large pools of bound calcium are sequestered in the ER of cells even thought the $(Ca)_i$ concentration is only 30 to 40 nM. In response to a variety of intracellular signals, such as inositol 1,4,5-triphosphate (IP3), an elevation of intracellular Ca^{2+} $(Ca)_i$ to several hundred nMs occurs. The elevation is usually transitory, however; the Ca^{2+} is rapidly pumped out of cells via the plasma membrane Ca^{2+}-ATPase pumps or back into the ER via its SERCA pump. Furthermore, the ER-sequestered Ca^{2+} is constantly leaking out into the cytoplasm. The sequestered store of Ca^{2+} is constantly replenished, however, by the SERCA pumps ability to transport the cytoplasmic Ca^{2+} back into the ER. Thus, inhibition of the SERCA pumps results in a threefold to fourfold elevation of $(Ca)_i$ (without any requirement for IP3 production). This primary elevation of $(Ca)_i$ leads to a depletion of the ER-Ca^{2+} pool and, in many cell types, results in generation of a signal that induces a change in the plasma membrane permeability to extracellular divalent cations, particularly Ca^{2+}. The initial intracellular discharge of the ER-sequestered calcium pools leads to an influx of extracellular Ca^{2+}, in keeping with the prediction from the capacitance model of Ca^{2+} entry, resulting in a secondary elevation in the $(Ca)_i$ and to activate programmed cell death in androgen-independent prostate cancer cells.

In cells treated with the TG bi-phasic changes in intracellular free calcium have been observed. After an initial increase from baseline values of 20-40 nM to values of 200-400 nM, induced by the emptying of the endoplasmic reticulum calcium pool, intracellular calcium return to baseline values within 6 to 18 hours. This decrease is mediated by activation of the calmodulin-dependent calcium pump of the plasma membrane since cells microinjected with a calmodulin inhibiting peptide maintained an elevated calcium . This first rise can be attenuated by intracellular buffers such as BAPTA or the calcium binding protein calbindin. In all dying cells a sustained second elevation of intracellular free calcium from a baseline of 20 -40 nM to 10- 50 μM has been observed. This rise to μmolar values precedes the morphological changes associated with apoptosis in both prostate and breast cancer cells. This second rise is asynchronous within the cell population but ultimately occurred in every dying cell. The proportion of cells showing a second increased per unit of time correlates with the number of cells showing DNA fragmentation and the proportion of cells showing loss of viability when measured by clonogenic assay. These results demonstrate the critical role of sustained elevations of intracellular Ca2+ in the programmed cell death induced by TG.

Chronic exposure of each cancer cell line to 500 nM TG was found to arrest the cells in the G_0/G_1 phase of the cell cycle within 24 hours. Analysis of mRNA expression of genes previously shown to be enhanced during androgen ablation-induced programmed cell death of normal prostate cells (e.g. calmodulin, TRPM-2, etc) showed that TG treatment of androgen-independent prostate cancer cells also leads to epigenetic reprogramming of the cells. Within 1 hour of TG treatment, androgen-independent cancer cells had elevated expression of additional genes, including glucose-regulated protein (GRP)B78, c-myc, and growth arrest and DNA damage (GADD)-153. Many of these

enhancements were acute, with expression decreasing by 24 hours of treatment. After a 24-hour lag period, the cells began to fragment their DNA (to sizes <300 kb); by 96 hours, 95% or more had fragmented their DNA regardless of cell line tested. Quantitative analysis of the DNA showed the nucleosomal ladder pattern of fragmentation characteristic of programmed cell death.

Prostate cancer cells must progress through the proliferative cell cycle in order for antiproliferative agents such as 5-fluorodeoxyuridine to induce programmed cell death, whereas proliferation is not required for TG-induced apoptosis. Furthermore, there are major differences in gene expression during the proliferation-independent programmed death induced by TG and the proliferation-dependent apoptosis associated with 5-FrdU. In additional studies, primary cultures of human prostate cancer cells were made. These cultures initially grow exponentially. During this exponential phase treatment with equal concentrations of either the cell proliferation-dependent chemotherapeutic agents 5-FrdU or doxorubicin or thapsigargin resulted in sterilization of culture dishes. In contrast, these cells were shown to go out of cycle and enter the proliferatively quiescent G_0 state after an initial proliferation period of approximately 10 days. The cultures were maintained for more than 6 weeks, with a spontaneous rate of cell death of ~2% /day. When these stationary cultures were exposed for 1 week to effective doses (i.e.100 nM) of doxorubicin or 5-FrdU, there was no significant activation of PCD either morphologically by videomicroscopic evaluation or quantitatively by DNA fragmentation . In contrast, exposure of the stationary cultures to 100 nM TG resulted in morphologic changes within 24 to 48 hours, with loss of ~ 85% of cells by day 4 of exposure. Thus, TG can induce programmed cell death of proliferatively quiescent G0 human prostate cancer cells without requiring their entry into or progression through the cell cycle.

Preliminary Data with TG Analogs

On the basis of these preclinical studies, it would appear that TG represents an excellent choice for treatment of prostate cancer because of its ability to kill prostate cancer cells in a proliferation-independent manner. Unfortunately, while TG is highly effective in inducing the proliferation independent programmed cell death of androgen independent prostate cancer cells, it is not cell type specific and is sparingly water soluble due to its high lipophilicity. In order to target TG's cytotoxicity specifically to prostate cancer cells systemically, TG must be chemically modified to produce an analog that can be coupled to a water-soluble prodrug carrier. This modification involves the introduction of a primary amine containing side chain into the TG molecule that can be coupled via a peptide bond to the carboxyl group of the C-terminal amino acid. In this way, TG can be targeted specifically to metastatic deposits of androgen independent prostate cancer producing enzymatically active PSA.

As a fellow in Dr. Isaacs laboratory I began collaboration with Dr. S. Brogger Christensen, Professor of Medicinal Chemistry at the Royal Danish School of Pharmacy, Copenhagen, Denmark. Dr. Christensen originally isolated and chemically characterized TG. Based on a model of the TG binding site within the SERCA pump it was determined that modifications of the TG molecule could possibly be made in the side chain in the 8-position without adversely effecting SERCA pump inhibitory activity. These modifications consist of de-esterifying TG in a position 8 and re-esterifying with side chains ending in a primary amine to allow for coupling via an amide bond to the C-terminal carboxylic acid of the PSA hydrolyzable peptide carrier, HSSKLQ. Using this rationale a series of TG analogs (i.e. ~30) modified in the 8-position with primary amine containing side chains were synthesized. These analogs were characterized for their ability to inhibit the SERCA pump and elevate intracellular calcium. In addition, these analogs were assayed for cytotoxic activity against androgen independent human prostate cancer cells in vitro. The preferred analogs contained long hydrocarbon side chains and ended in a primary amine (figure 1). The rationale for the design of these analogs was that the longer linker would keep the large TG molecule out f the PSA catalytic site. One of theses analogs, containing a 12-amino dodecanoate side chain (12ADT) was found to have an IC_{50} value against TSU cells of ~ 500 nM (figure 2). This analog was coupled to the PSA-peptide carrier and once again no hydrolysis was observed when incubated with enzymatically active PSA.

From my earlier work with the doxorubicin prodrugs and the more recent results with the TG analog prodrugs, it was apparent that an amino acid was required in the P_{-1} position of the prodrug (i.e. HSSKLQ-P_{-1}AA). When the P_{-1} amino acid was leucine, PSA-hydrolysis was observed with both doxorubicin and the APT analog. Therefore, leucine was coupled to several TG analogs. In this way, a TG analog consisting of leucine coupled to a 12-carbon (i.e. aminododecanoic acid) side chain was produced [i.e. Leu-aminododecanoic-TG (L-12ADT)] (figure 2). The rationale for this compound was that the increase in lipophilicity secondary to the long 12-carbon side chain would increase

plasma membrane permeability to compensate for the positively charged leucine. The L-12ADT analog has an IC50 value of 75 +/- 5 nM against TSU cells in clonogenic survival assays (figure 2). This level of activity was similar to the parent TG, making this the most potent TG analog developed to date. On the basis of the in vitro studies, the L12ADT represents the best TG analog to date. Therefore, the L12ADT was coupled to the HSSKLQ peptide carrier to produce HSSKLQ-L12ADT, figure 4. HPLC analysis demonstrated that PSA could liberate L12ADT from the peptide with a Km of 470 μM, table 2. In cytotoxicity assays in vitro, the LD_{50} against PSA-producing LNCaP cells was 74 ± 3 nM while against non-PSA producing TSU cells the LD_{50} was 2130 ± 320 nM (i.e.~30-fold difference in cytotoxicity in presence vs. absence of enzymatically active PSA), table 3.

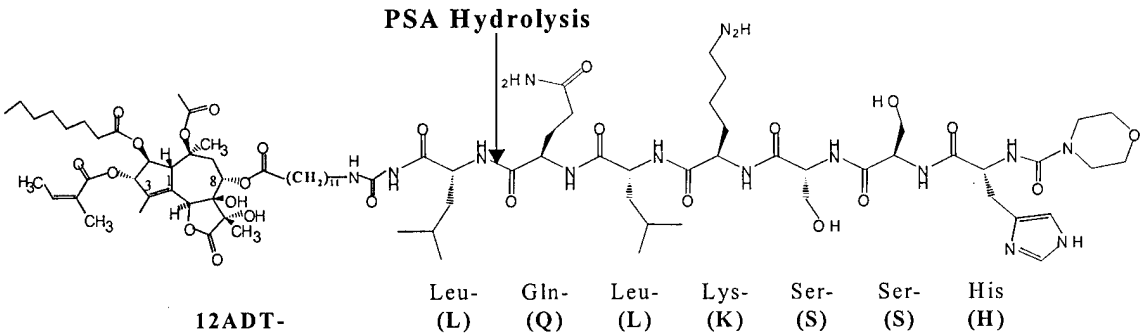

Figure 4. Chemical structure of the Mu-HSSKLQ-L12ADT prodrug. The site of PSA hydrolysis is indicated.

On the basis of the in vitro studies, the L12ADT represents the best TG analog to date. Therefore, the L12ADT was coupled to the HSSKLQ peptide carrier to produce HSSKLQ-L12ADT, figure 4. HPLC analysis demonstrated that PSA could liberate L12ADT from the peptide with a Km of 470 μM, table 2. In cytotoxicity assays in vitro, the LD_{50} against PSA-producing LNCaP cells was 74 ± 3 nM while against non-PSA producing TSU cells the LD_{50} was 2130 ± 320 nM (i.e.~30-fold difference in cytotoxicity in presence vs. absence of enzymatically active PSA), table 3.

Progress Over the Last 12 months of Funding

In Vivo Activity of HSSKLQ-L12ADT prodrug

These in vitro studies demonstrated that PSA could hydrolyze the HSSKLQ-L12ADT to produce cytotoxic levels of L12ADT. Therefore, sufficient quantities of the prodrug were synthesized to allow preliminary toxicity studies and to assess antitumor effect. In initial studies, mice were given a single intravenous injection of the prodrug and the LD_{100} was determined to be ~ 20,000 nmoles/kg. In addition, mice have also been given doses of HSSKLQ-L12ADT of 50 mg/kg/week for 6 weeks via Alzet minipump without significant toxicity or weight loss. Mice were also treated with daily subcutaneous injections of the HSSKLQ-L12ADT prodrug at a dose of 7 mg/kg for three weeks without significant toxicity. These studies demonstrate that the peptide carrier can neutralize the systemic toxicity of the TG analog and indicate that significant nonspecific cleavage of the prodrug does not occur.

In *in vivo* efficacy studies, a series of nude mice were inoculated subcutaneously with PSA-producing LNCaP cells. After ~ 3weeks, tumor sizes were measured and serum PSA levels were determined and mice divided into two groups having equal starting tumor sizes. One group of animals received a subcutaneous Alzet osmotic minipump containing sufficient HSSKLQ-L12ADT prodrug in 10% DMSO/sterile water to deliver 50 mg/kg/week. A second group received a similar Alzet osmotic minipump containing only vehicle. The response of the two groups over a 40 day period is presented in figure 5. These data demonstrated a nearly complete cessation of net growth during the period of observation. As an additional control a size-matched group of animals bearing the non-PSA producing human renal cell carcinoma cell line SN12C were treated with the same dose of prodrug via Alzet minipump. In this group, there was no demonstrable effect on SN12C growth in the TG-prodrug treated group compared to control, figure 5. These results confirm that enzymatically active PSA must be present within the tumor for prodrug activation and antitumor effect.

In a second study, another group of LNCaP bearing animals were given daily subcutaneous injections of the prodrug (7 mg/kg/day) for a total of 50 mg/kg/week for 3 weeks. In this group there was a > 60 ± 8% decrease in tumor weight in treated animal vs. vehicle controls ($p < 0.005$). No significant toxicities were observed in either treated group. These results suggest that chronic infusion of the TG prodrug may be the preferred method of delivery. Additional studies are now underway to confirm these exciting results.

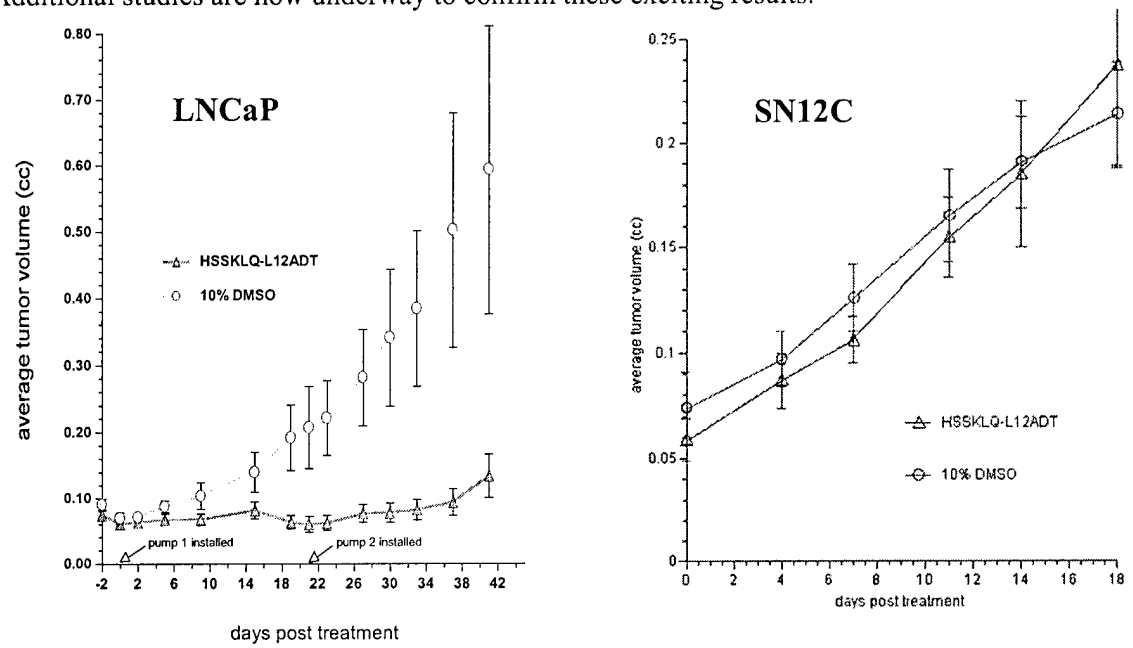

Figure 5. In vivo activity of HSSKLQ-L12ADT against PSA producing LNCaP and non-PSA producing SN12C xenografts. Tumor bearing animals were treated with 50 mg/kg/week of prodrug via osmotic minipump. Controls received vehicle (10% DMSO) also via minipump. Eight animals were treated per group.

Ranking of the TG analog on the basis of cytotoxicity and hydrolysis from the peptide carrier by PSA.

Studies to date with both the doxorubicin prodrugs and the TG analog prodrugs have indicated that PSA requires an amino acid in the P-1 position for hydrolysis to occur. Originally, the amino acid leucine was chosen as the linker between the peptide and the TG analog on the basis of studies with the HSSKLQ-L-Dox doxorubicin prodrug. Recently a series of additional amino acid-TG analogs were synthesized in which the leucine moiety was replaced by other amino acids, specifically alanine (A), serine (S), or phenylalanine (F). In SERCA inhibition assays using microsomes isolated from rabbit skeletal muscle, the concentrations to inhibit 50% of the SERCA pump activity (i.e. IC_{50} values) for these amino acid analogs are almost equivalent (42). This result was as predicted from the energy minimalization model of TG inhibition of the SERCA pump in which the 8-position of the molecule is not involved in binding to the protein but instead projects out of the binding pocket. These additional amino acid analogs have also been screened for cytotoxicity against the LNCaP and TSU cancer cell line and have LD_{50} values that are 1.5-10 fold higher than the L12ADT (i.e. 30 nM vs. 200-900 nM), table 3 (42). In addition, in an attempt to produce a prodrug that was potentially more stable in serum, the *d*-leucine analog (*d*-L12ADT) was also synthesized and found to have an identical LD_{50} as *l*-L12ADT (42).

To date, four additional prodrugs have been synthesized and assayed for PSA hydrolysis. All of these prodrugs are hydrolyzed by PSA. The kinetics of hydrolysis for each of these prodrugs is summarized in table 2. The data were analyzed by Lineweaver-Burke reciprocal plots to determine the Michaelis-Menten constant (i.e. K_m) expressed as the amount of substrate needed to saturate half of the enzyme and the catalytic rate constant (i.e. k_{cat}) expressed as the amount of substrate converted to product per time per amount of enzyme. Since the k_{cat} value reflects the efficiency of the conversion of the substrate to products per enzyme while the K_m value reflects the concentration

dependence for enzymatic activity, higher k_{cat}/K_m ratio values denote better substrates. Therefore, these ratios were calculated in order to compare PSA hydrolysis of the various prodrugs.

The HSSKLQ-S12ADT prodrug appears to be most efficiently hydrolyzed by PSA. In contrast, the HSSKLQ-*d*-L12ADT prodrug is not hydrolyzed at all by PSA. Each prodrug was tested against the PSA-producing LNCaP cells and also against PSA non-producing TSU cells. These results are summarized in table 3. Prodrugs of the L-12ADT and S-12ADT analogs have comparable activity against PSA-Producing LNCaP cells, table 3. The S-12ADT prodrug, however, is ~ 140 fold less toxic to non-PSA producing TSU cells compared to the L-12ADT prodrug which is ~ 30 fold less toxic. The S-12ADT prodrug, therefore, has the highest in vitro therapeutic index of the prodrugs tested to date.

Table 2. Kinetics of TG prodrug hydrolysis by PSA

TG Analog	Km (μM)	k_{cat} (1/s)	k_{cat}/Km (1/s*M)
Mu-HSSKLQ-L12ADT	473	.00962	21.87
Mu-HSSKLQ-A12ADT	3155	.2332	74.86
Mu-HSSKLQ-S12ADT	221	.0984	445.23
Mu-HSSKLQ-F12ADT	ND	ND	ND

ND= Kinetics for Mu-HSSKLQ-F12ADT could not be calculated due to its poor solubility above 100μM.

Cytotoxicity of Amino Acid Analogs

[LD_{50} (nM) Clonogenic Survival]

	PSA-Producing LNCaP Cells		Non-PSA Producing TSU Cells		Fold-Difference
TG Analog	HSSKLQ Prodrug	Free Drug	HSSKLQ Prodrug	Free Drug	Prodrug Activity TSU/LNCaP
L-12ADT	74 ± 3	13 ± 3	2130 ± 320	30 ± 10	29
A-12ADT	320 ± 8	3 ± 2	3930 ± 130	280 ± 60	12
S-12ADT	55 ± 8	38 ± 4	7750 ± 45	895 ± 55	140
F-12ADT	118 ± 13	3 ± 1	342 ± 40	210 ± 20	2.8

Table 3. Cytotoxicity of several amino acid containing TG analogs against PSA-producing LNCaP cells and non-PSA producing TSU cells

Pharmacokinetic Studies using the HSSKLQ-L12ADT prodrug

To determine clearance rates and serum half-life of the prodrug in mice and to document whether the prodrug underwent significant hydrolysis to free L12ADT in serum, a sensitive method was developed to measure these two compounds in mouse serum. To accomplish this analysis, a series of mice were given the maximally tolerated single intravenous dose of the prodrug and these mice were then sacrificed at various time points (i.e. 5, 10 , 30 minutes and 1, 3, 6, 12, 24 hr) and serum obtained. Serum proteins were precipitated with acetonitrile and supernatants were then dried under nitrogen stream and resuspended in 0.1% formic acid/acetonitrile. Spiked serum samples that underwent a similar extraction procedure were used as calibration standards. Standards and samples were analyzed by liquid chromatography coupled to a quadripole mass spectrometer (LC/MS/MS) [PESciex API 3000]. This LC/MS/MS machine is available as a shared core facility of the Departments of Urology and Oncology and is used routinely by our laboratories for these analyses. A multistep gradient elution HPLC method was developed to separate both the

HSSKLQ-L12ADT prodrug and the L12ADT analog with eluent A= 2mM ammonium acetate with 0.1 % formic acid and eluent B= 90% acetonitrile/10% deionized water.

Flow from the column was directed into the PE Sciex API3000 LC/MS/MS and indvidual compounds were detected by looking for the following transitions. For the HSSKLQ-L12ADT prodrug there was a lack of fragmentation and the +2 ion was most abundant in the MS and MS/MS conditions at 843.5 m/z. Because of this the parent/parent transition was measured with the MRM being 843.5/843.5. For the L12ADT the transition of maximum sensitivity was found to be an 891.5 parent M+1 with a 216.4 daughter ion and therefore the measured transition was 891.5/216.4 m/z (fragmenation achieved using an ion spray source with heated nebulizer at 350 °C and 4L/minute desolvation). The peak corresponding to the 843.5/843.5 transition in the extracted chromatogram has a retention time of 6.14 minutes and the 891.5/216.4 transition peak elutes at 9.06 minutes. Calibration was done using extracted standards in a range of 0.001-100 μM and linear regression analysis used to generate best fit line from which peak areas of samples were converted to concentration of prodrug or L12ADT.

Following an injection of the maximally tolerated single intravenous dose of 7 mg/kg, the concentration of prodrug at 10 minutes post injection was 65 ± 8 μM. By 24 hrs the serum concentration had declined to 90 ± 7 nM. The serum half-life of the HSSKLQ-L12ADT prodrug was 2.4 hours. In contrast, nanomolar levels of the L12ADT prodrug were observed with a peak L12ADT concentration of 100 nM at 6 hrs and a concentration of 2 nM at 24 hrs. These data demonstrate that only a small percentage (i.e. < 1%) of the prodrug becomes hydrolyzed to the free L12ADT over a 24 hr period. In contrast, the doxorubicin prodrug being developed by Merck is significantly hydrolyzed in both mouse and human serum (i.e. ≥ 33% hydrolysis to free doxorubicin by 24 hr) (47).The data also demonstrate that serum prodrug levels remain above the LD_{50} for PSA-producing cells (i.e. 75 nM) for 24 hrs following a single IV dose. Further studies will be performed as outlined in specific aim 2 to determine normal tissue and tumor levels of the TG prodrugs and free TG analogs at various time points following IV dosing.

Proposed Studies for the 2002 funding period

To date amino acid containing TG analogs have been identified that are potent cytotoxins. These TG analogs are readily coupled to peptides. On the basis of these characteristics, I do not believe it is necessary to produce any additional TG analogs and will utilize these two TG analogs for further prodrug development. To date we have also identified the prodrug HSSKLQ-L12ADT as our prototype compound. Recently we have generated additional data to suggest that other amino acid substitutions (e.g.HSSKLQ-S12ADT) produce prodrugs that have improved PSA hydrolysis kinetics and have similar activity against PSA-producing prostate cancer cells. Therefore, there are two major goals to be accomplished during the 2002 funding period. The first is to further optimize the PSA peptide carrier to identify the best PSA substrates on the basis of enzyme kinetics and specificity of hydrolysis. The second goal is to produce prodrugs by coupling these peptides to the TG analogs L-12ADT and S-12ADT and determine their in vitro cytotoxicity. From these studies, we will select the best prodrug on the basis of efficacy and potency that will be carried forward for additional in vivo studies outlined in specific aims 2 and 3.

To accomplish the first goal we will test additional PSA sequences based on preliminary data with a number of new PSA peptide sequences. Using a membrne bound peptide library, I have screened a series of additional peptides consisting of variations in the lead HSSKLQ peptide. These preliminary studies have identified sequence motifs that could represent better PSA substrates than the lead HSSKLQ peptide. Over the next year the peptides listed below will be synthesized. This series include both the peptides that were best hydrolyzed by PSA from the cellulose membranes as well as several consensus sequences based on the hydrolysis data. Initially we will evaluate the first six amino acids (i.e. P1-P6). These peptides will be synthesized as we have previously described using the solid phase peptide synthesizer available in our laboratory using standard Fmoc coupling chemistry. Each peptide will be acetylated to protect the N-terminal amine. The fluorophore 7-amino-4-methylcoumarin (AMC) will be coupled to the C-terminal carboxyl of each peptide. The AMC peptides are synthesized because they can be rapidly screened for hydrolysis by PSA and other extracellular proteases in a 96 well fluorescent plate reader used routinely in our laboratory.

The peptides to be synthesized are as follows (Note lowercase represents *d*-amino acid):

Sequences from Table 4,5				Consensus Sequences
1. GASKLQ	(2P)	5. GSSKYQ	(21P)	9. GAAKYQ
2. GSAKLQ	(3P)	6. GKSQYQ	(22P)	10. Gs AKYQ
3. Gs SKLQ	(5P)	7. GKSSYQ	(23P)	11. GKAQY
4. GSs KLQ	(6P)	8. GSSKLH	(24P)	12. GAAKYH

Each of these substrates will be tested for efficiency of PSA hydrolysis by determining the Km and kcat values as described previously. In addition, each substrate will be tested for specificity of hydrolysis using a panel of commercially available purified extracellular proteases that include chymotrypsin, elastase, trypsin, plasmin, thrombin, urokinase, tissue kallikrein (hK1), and hK2 as described previously. Each substrate will also be tested for stability in human and mouse plasma and serum as described previously. The substrates that possess the best combination of efficient PSA hydrolysis (i.e. high kcat/Km ratio), specificity vs. extracellular proteases and serum stability will be selected for further analysis.

To accomplish the second goal of specific aim 1, we will couple the best peptide substrates identified above to L-12ADT and S-12ADT. These new prodrugs will be tested for PSA hydrolysis, hydrolysis by other extracellular protease and for stability in human serum. Those prodrugs that are specifically hydrolyzed by PSA and stable in human serum will be tested in vitro against PSA-producing LNCaP and LAPC-4 cells to determine cytotoxicity and against non-PSA producing TSU and SN12C cells to determine specificity of cell killing. In these assays these new prodrugs will be compared "head to head" with our current prototype prodrug HSSKLQ-L12ADT. We will select the best 3-5 prodrugs based on equivalent or improved characteristics (i.e. PSA hydrolysis, serum stability, in vitro cytotoxicity) compared to the current lead prodrug and these will be further tested in vivo against PSA-producing LNCaP xenografts in order to identify the optimal prodrug that can be moved forward into clinical trials

KEY RESEARCH ACCOMPLISHMENTS:

1. Synthesized primary amine containing TG analogs that are potent cytotoxins
2. Demonstrated that this leucine containing TG analog (L-12ADT) can be coupled to a PSA-specific peptide and be hydrolyzed free from the peptide by enzymatically active PSA.
3. Demonstrated that this prodrug is relatively inactive against PSA non-producing cancer cells while ~ 30 fold enhancement of therapeutic effect occurs in the presence of PSA.
4. Demonstrated the in vivo efficacy of this HSSKLQ-L12ADT prodrug against PSA producing xenografts
5. Developed a method to determine pharmacokinetics of HSSKLQ-L12ADT using LC-MS.
6. Synthesized and tested additional amino acid containing TG analogs and determined enzyme kinetics.
7. Screened a series of additional PSA peptides substrates in attempt to optimize PSA sequence

REPORTABLE OUTCOMES:

Manuscripts, abstracts, presentations;

1. **Denmeade, S.R.**, Lovgren J., Khan, S.R., Lilja, H., Isaacs, J.T. Activation of Latent Protease Function of Pro-HK2, but not Pro-PSA, Involves Auto-Processing. Prostate, 48:122-126, 2001.

2. **Denmeade, S.R.**, Sokoll, L.J., Chan, D.W., Khan, S.R., Isaacs, J.T. Concentration of Enzymatically Active Prostate-Specific Antigen (PSA) in the Extracellular Fluid of Primary Human Prostate Cancers and Human Prostatic Cancer Xenograft Models. Prostate, 48:1-6, 2001.

3. Jakobsen, C.M., **Denmeade, S.R.**, Isaacs, J.T., Gady, A.M., Olsen, C.E., Christensen, S.B. Design, Synthesis and Pharmacological Evaluation of Thapsigargin Analogues for Targeting Apoptosis to Prostatic Cancer Cells. J. Med. Chem., 44: 4696-4703, 2001.

Presentations:

1. "PSA-Based Prodrug Therapy for Androgen Independent Prostate Cancer", CaPCURE Annual Meeting, Lake Tahoe, NV.
2. "PSA-Activated Prodrugs/Protoxins as Targeted Therapy for Prostate Cancer", AACR Special Meeting on Prostate Cancer, Naples, FL.

Funding Applied for and Received:

1. Special Project on Research Excellence (SPORE) 2P50 CA58236-07: Prostate Cancer (Developmental Funds for one year).

2. Special Project on Research Excellence (SPORE) 5P50 CA88843-01: Breast Cancer (Career Developmental Funds for one year).

3. Department of Defense Prostate Cancer Research Program Idea Development Award (Funding to begin in 2002).

Patents and Licenses applied for and/or issued;

"Tissue Specific Prodrug", Inventors: Isaacs, JT, **Denmeade, SR**, Christensen, SB, Lilja H. United States Patent Number 6,265,540 (filed 5/19/97 and 3/30/98). This patent refers to the PSA-specific peptide, the creation of PSA targeted prodrugs, and primary amine containing TG analogs.

"Activation of Peptide Prodrugs by Human Glandular Kallikrein 2 (hK2)", Inventors: Isaacs, JT, **Denmeade, SR** , Lilja H. Patent filed 7/00. This invention refers to development of peptide substrates for the protease hK2, the creation of hK2 targeted prodrugs, and also refers specifically to the TG analog L-12ADT.
Funding applied for based on work supported by this award;

CONCLUSIONS:

The original hypothesis of the proposal was that the proteolytic activity of PSA, which is highly expressed by androgen independent prostate cancer cells, could be used to activate prodrugs specifically to liberate a cytotoxic agent at sites of metastatic prostate cancer. In the original proposal, I had intended to use proteasome inhibitors as the preferred cytotoxic agent. Preliminary studies demonstrated that this approach might not be feasible due to instability of the proteasome inhibitors and poor cell penetration. In addition, the pharmaceutical company that I was collaborating with decided not to continue this work, citing lack of funds.

Instead of abandoning the project at that early point, I decided to continue the project and use a different cytotoxic warhead by substituting a TG analog for the proteasome inhibitor. In doing so, I was building on the work with TG that I began as a post-doctoral fellow. TG represents an ideal treatment for slowly proliferating prostate cancers as it can induce apoptosis in a proliferation independent manner. To develop the TG-based prodrug I have followed the same task list and timetable as that outlined in the original proposal. For the renewal year I intend to complete tasks 3 and begin task 4 of the original proposal, substituting TG analogs for proteasome inhibitors as the cytotoxic agent that will be coupled to the PSA-specific peptide.

The preclinical data with the TG analog L12ADT (figure 2,3) demonstrate that it is a potent cytotoxin against prostate cancer cell lines. When coupled to the PSA-specific peptide, the prodrug HSSKLQ-L12ADT is readily hydrolyzed by PSA. This prodrug is relatively inactive in vitro in the absence of enzymatically active PSA in the media; however, when PSA is present the activity increases ~ 30 fold. In vivo, effective and specific antitumor activity is observed with the HSSKLQ-L12ADT at doses that are non-toxic to the host animal. Additional TG prodrugs have also been screened and demonstrate enhanced PSA hydrolysis. Over the next funding period, additional PSA prodrugs that have improved hydrolysis kinetics and antitumor efficacy in vitro will be synthesized and tested in vivo against PSA-producing and non-producing xenografts to select a lead prodrug that will be carried forward for testing in clinical trials.

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13. **Denmeade, S.R.,** Sokoll, L.J., Chan, D.W., Khan, S.R., Isaacs, J.T. Concentration of Enzymatically Active Prostate-Specific Antigen (PSA) in the Extracellular Fluid of Primary Human Prostate Cancers and Human Prostatic Cancer Xenograft Models. Prostate, 48:1-6, 2001.
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Appendices:

Design, Synthesis, and Pharmacological Evaluation of Thapsigargin Analogues for Targeting Apoptosis to Prostatic Cancer Cells

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A series of thapsigargin (TG) analogues, containing an amino acid applicable for conjugation to a peptide specifically cleaved by prostate-specific antigen (PSA), has been prepared to develop the drug-moiety of prodrugs for treatment of prostatic cancer. The analogues were synthesized by converting TG into *O*-8-debutanoylthapsigargin (DBTG) and esterifying *O*-8 of DBTG with various amino acid linkers. The compounds were evaluated for their ability to elevate the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in TSU-Pr1 cells, their ability to inhibit the rabbit skeletal muscle SERCA pump, and their ability to induce apoptosis in TSU-Pr1 human prostatic cancer cells. The activity of analogues, in which DBTG were esterified with ω-amino acids [HOOC-$(CH_2)_n NH_2$, $n = 5-7$, 10, 11], increased with the linker length. Analogues with 3-[4-(L-leucinoylamino)phenyl]propanoyl, 6-(L-leucinoylamino)hexanoyl, and 12-(L-serinoylamino)dodecanoyl were considerably less active than TG, and analogues with 12-(L-alaninoylamino)dodecanoyl and 12-(L-phenylalaninoylamino)dodecanoyl were almost as active as TG. The 12-(L-leucinoylamino)dodecanoyl gave an analogue equipotent with TG, making this compound promising as the drug-moiety of a PSA sensitive prodrug of TG.

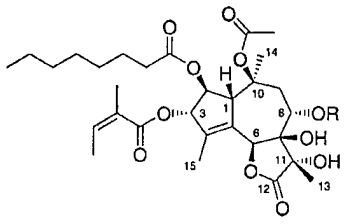
Introduction

Thapsigargin (TG, **1**) (Chart 1) is a sesquiterpene-γ-lactone isolated from seeds and roots of the umbelliferous plant, *Thapsia garganica* L.[1,2] TG selectively inhibits the ubiquitous sarcoplasmic and endoplasmic reticulum Ca^{2+}-dependent ATPases (SERCA's) with an apparent dissociation constant of 2.2 pM or less.[3,4] TG induced inhibition of the SERCA pump leads to depletion of the ER Ca^{2+} pool and a capacitance influx of extracellular Ca^{2+} resulting in a sustained elevation (i.e., 200−400 nM) of the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$).[5] This sustained elevation of $[Ca^{2+}]_i$ subsequently leads to DNA fragmentation and programmed cell death (apoptosis) of treated cells.

TG induces apoptosis in rat (AT-3) and human (TSU-Pr1, PC-3, DU-145) androgen-independent prostatic cancer cell lines with LC_{50} values for cell death in the 10−100 nM range.[5] This TG induced apoptosis does not require the cells to be in proliferative cell cycle but can be induced in primary human prostatic cancer cell cultures in which about 98% of the cells are out of cycle in G_0.[6] These studies have identified the SERCA pump as a new therapeutic target for activating apoptosis of androgen-independent prostatic cancer cells.

TG's ability to kill proliferatively quiescent G_0cel ls by inhibiting the ubiquitous SERCA's means that it will be difficult to administer TG systemically as a therapeutic agent without significant host toxicity. One approach to specifically target TG cytotoxicity to prostatic cancer cells is to take advantage of the unique secretion of prostate-specific antigen (PSA) by these cells. PSA is a serine protease with chymotrypsin-like substrate specificity that is enzymatically active only in the extracellular fluid of prostatic cancer cells, whereas it is enzymatically inactivated in blood serum.[7−9] Previously a highly specific and efficient PSA substrate with the sequence His−Ser−Ser−Lys−Leu−Gln-(HSSKLQ) was identified.[9] If TG is converted to *O*-8-debutanoylthapsigargin (DBTG, **2**) (Chart 1) and DBTG is esterified in the *O*-8 position with an amino carboxylic acid linker, the resulting TG analogue can be coupled to the C-terminal glutamine (Q) of this peptide to form a peptide bond that is hydrolyzable by enzymatically active PSA (Figure 1). Such a TG analogue prodrug will be cleaved only in the extracellular fluid of PSA-

Chart 1[a]



TG (**1**), R = $CO(CH_2)_2CH_3$

DBTG (**2**), R = H

[a] Structures of thapsigargin (TG, **1**) and *O*-8-debutanoylthapsigargin (DBTG, **2**).

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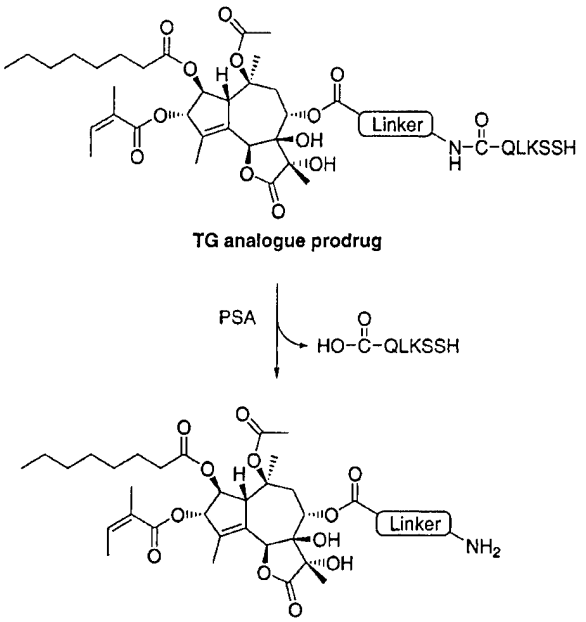


Figure 1. Prostate-specific antigen (PSA) releases the active TG analogue from the TG analogue prodrug by hydrolysis of the peptide promoiety (HSSKLQ).

secreting prostatic cancer cells thus specifically targeting the TG analogue cytotoxicity to prostatic cancer cells.

In a previous study,[10] we reported on the synthesis and apoptotic activity of a series of *O*-8 substituted aromatic amine TG analogues (**3c,e,f** and **4a−e**) (Chart 2). Analogues **4c−e** were the most potent, being 1.5, 1.7, and 1.7 times less potent inhibitors of the rabbit skeletal muscle SERCA pump than TG, respectively, and 9, 4, and 8 times less cytotoxic against TSU-Pr1 human prostatic cancer cells, respectively.

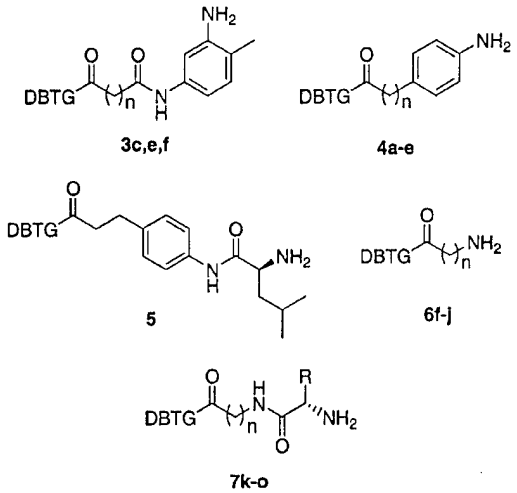
The goal of this paper was to investigate the structure−activity relationship of TG analogues, in which the *O*-8 butyric acid has been substituted with an ω-amino acid (analogues **6f−j**) (Chart 2) or an α-amino acid conjugated ω-amino acid (analogues **5** and **7k-o**) (Chart 2).

All target compounds were evaluated for their ability to elevate $[Ca^{2+}]_i$ in TSU-Pr1 cells, their ability to inhibit the rabbit skeletal muscle SERCA pump, and their ability to kill TSU-Pr1 human prostatic cancer cells.

Chemistry

DBTG (**2**) was prepared from TG (**1**) by selective trans-esterification of the *O*-8-butanoyl ester in methanol using triethylamine as catalyst.[11,12] Analogue **5** was synthesized as shown in Scheme 1. 4-Amino-*trans*-cinnamic acid hydrochloride was hydrogenated and the carboxyl group protected as the methyl ester to give **8**. N_α-Boc-L-leucine was coupled[13] to the aromatic amine to give **9**. Deprotection of the carboxylic acid gave **10**, coupling to **2** gave **11**, and deprotection of the amine gave analogue **5**. Analogues **6f−j** were synthesized as outlined in Scheme 2. Coupling of the *N*-Boc protected aliphatic amino acids **12f−j** with **2** gave **13f−j**, and deprotection of the amino group gave **6f−j**. Analogues **7k−m** were synthesized as shown in Scheme 3. N_α-Boc-L-leucine was coupled to methyl esters **14f,j** to give **15k,l**, and N_α-Boc-L-alanine was coupled to methyl ester **14j** to give **15m**. Deprotection of the carboxylic acid gave **16k−m**, coupling to **2** gave **17k−m**, and deprotection of the amine gave **7k−m**. Analogues **7n,o** were synthesized as outlined in Scheme 4. N_α-Boc-L-serine and N_α-Boc-L-phenylalanine were coupled to analogue **6j** to give **18n,o**, and deprotection of the amine gave **7n,o**.

Chart 2[a]



a: n = 0
b: n = 1
c: n = 2
d: n = 2, *trans* C=C
e: n = 3
f: n = 5
g: n = 6
h: n = 7
i: n = 10
j: n = 11
k: n = 5, R = $CH_2(CH_3)_2$
l: n = 11, R = $CH_2(CH_3)_2$
m: n = 11, R = CH_3
n: n = 11, R = CH_2OH
o: n = 11, R = CH_2Ph

[a] TG analogues with amine-containing linkers esterified to the *O*-8 hydroxyl of **2**.

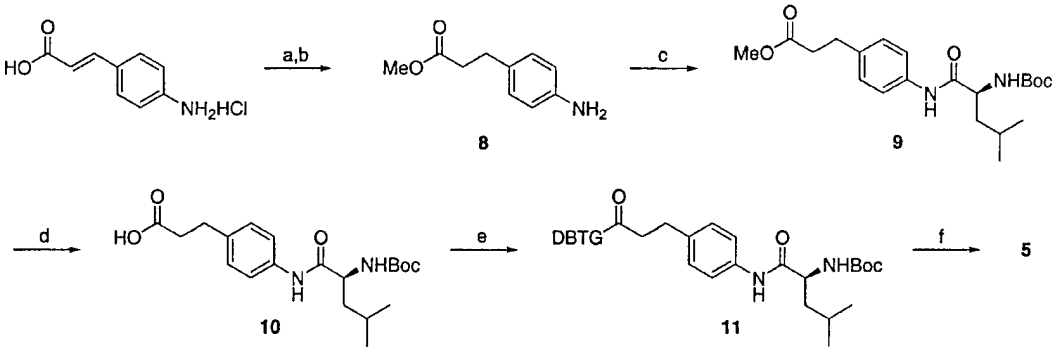
Pharmacology

SERCA containing microsomes were isolated from rabbit skeletal muscle by differential centrifugation of the muscle homogenate.[14,15]

The SERCA activity was measured with a coupled enzyme assay as the rate of ATP hydrolysis.[16,17] The activity at each dose (nmol/mg of SR protein) of TG analogue was expressed as percentage of the uninhibited control activity and was determined in triplicate. Inhibition curves were corrected for Ca^{2+}-independent (TG insensitive) ATPase activity by subtracting the residual activity at high inhibitor concentrations, which typically represented 10% of the total activity. The amount of TG analogue required to inhibit 50% of the maximal Ca^{2+}-dependent ATPase activity in 1 mg of SR protein was expressed as ID_{50} values, and was determined by 4-parameter curve-fitting (Table 1).

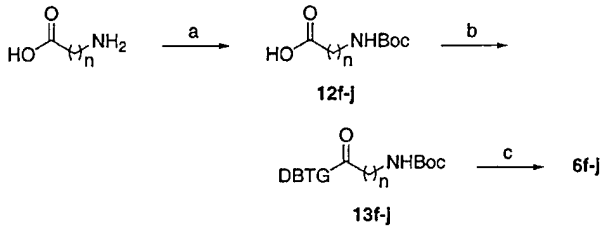
The apoptotic activity of each TG analogue against TSU-Pr1 human prostatic cancer cells was determined as previously described.[18] The apoptotic activity was expressed as the concentration of analogue LC_{50} (μM) capable of inducing 50% loss of clonogenic survival as compared to untreated controls (Table 1).

Scheme 1[a]



[a] Reagents: (a) Pd/C, H_2, 2-metoxyethanol; (b) MeOH, $SOCl_2$; (c) N_α-*tert*-butoxycarbonyl-L-leucine, hexachloroacetone, Ph_3P, pyridine, THF; (d) 2 M NaOH (aq), MeOH; (e) **2**, DCC, DMAP, CH_2Cl_2; (f) TFA, CH_2Cl_2.

Scheme 2[a]



[a] Reagents: (a) 5 M NaOH (aq), $(Boc)_2O$, *tert*-BuOH; (b) **2**, DCC, DMAP, CH_2Cl_2; (c) TFA, CH_2Cl_2.

The increase in cytosolic calcium concentration ($[Ca^{2+}]_i$) in TSU-Pr1 cells induced by TG analogues at effective cytotoxic concentrations was determined as previously described (Table 2).[10,19]

Results and Discussion

Previous published results concerning PSA activated doxorubicin prodrugs[20] promoted us to conjugate analogue **4c** with L-leucine to give analogue **5**. The conjugated analogue showed decreased SERCA inhibition and apoptotic activity (Table 1). The decreased activity of **5** was attributed to a decreased lipophilicity due to protonization of the α-amine at physiological pH. Previously, a positive correlation between lipophilicity and histamine-releasing activity was found in a series of *O*-2 and *O*-8 substituted TG analogues.[21]

The previously prepared aromatic analogues **3c,e,f** were 328, 81, and 4.4 times less potent SERCA inhibitors than TG, respectively, and >800, 533, and 103 times less cytotoxic, respectively.[10] Apparently, the potency also in this case increases with increasing lipophilicity. A similar structure–activity relationship was found in the series of aliphatic analogues **6f–j** (Table 1).

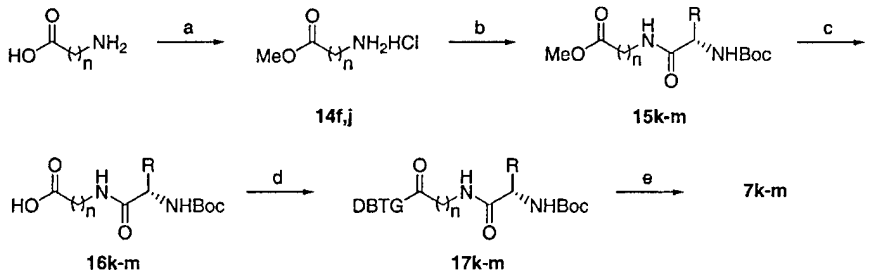
Table 1. ID_{50} (nmol/mg of SR protein) Values for Inhibiting 50% of Maximal Rabbit Skeletal Muscle SERCA Activity and LC_{50} (μM) Values for 50% Loss of Clonogenic Survival of Human Prostate Cancer TSU-Pr1 Cells[a]

compd	ID_{50} (nmol/mg SR protein)	activity relative to TG[b]	LC_{50} (μM)	activity relative to TG[c]
TG	13.4 ± 1.4	1	0.03 ± 0.004	1
5	466 ± 22	35	0.88 ± 0.04	29
6f	1332 ± 83	99	>20	>667
6g	1206 ± 57	90	10.92 ± 2.28	364
6h	223 ± 15	17	3.85 ± 0.21	128
6i	40 ± 3	3.0	0.75 ± 0.03	25
6j	35 ± 4	2.6	1.16 ± 0.16	39
7k	3842 ± 315	287	>20	>667
7l	45 ± 3	3.4	0.03 ± 0.01	1.0
7m	16.5 ± 1.6	1.2	0.28 ± 0.06	9.3
7n	10.3 ± 0.5	0.8	0.89 ± 0.04	30
7o	n.d.	-	0.21 ± 0.02	7.0

[a] Results expressed as mean ± standard deviation of triplicate measurements. [b] ID_{50} analogue/ID_{50} TG. [c] LC_{50} analogue/LC_{50} TG.

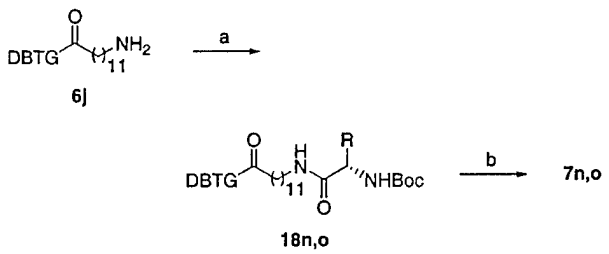
Conjugation of **6f** with L-leucine to give **7k** decreased the SERCA inhibitory activity, and the apoptotic activity was still poor (Table 1). In contrast, conjugation of **6j** with L-leucine to give **7l** only marginally changed the SERCA inhibition, and surprisingly increased the apoptotic activity affording an analogue equipotent with TG. Replacement of L-leucine in **7l** with L-alanine and L-phenylalanine (**7m,o**, respectively) only to a limited extent influenced the activity, whereas introduction of the more hydrophilic L-serine (**7n**) afforded a less apoptotic analogue.

Scheme 3[a]



[a] Reagents: (a) MeOH, $SOCl_2$; (b) N_α-*tert*-butoxycarbonyl-L-leucine or N_α-*tert*-butoxycarbonyl-L-alanine, DIPEA, DCC, CH_2Cl_2; (c) 2 M NaOH (aq), MeOH; (d) **2**, DCC, DMAP, CH_2Cl_2; (e) TFA, CH_2Cl_2.

Scheme 4[a]



[a] Reagents: (a) N_α-*tert*-butoxycarbonyl-L-serine or N_α-*tert*-butoxycarbonyl-L-phenylalanine, DCC, HOBT, DMF; (b) TFA, CH_2Cl_2.

Table 2. Increase in Cytosolic Ca^{2+} Concentration ($[Ca^{2+}]_i$) in TSU-Pr1 Cells at Effective Cytotoxic Concentrations[a]

compd	1000 nM $[Ca^{2+}]_i$ (nM)	100 nM $[Ca^{2+}]_i$ (nM)
5	451 ± 54	91 ± 16
6f	284 ± 51	NE[b]
6g	209 ± 6	NE[b]
6h	217 ± 20	NE[b]
6i	420 ± 3	242 ± 7
6j	369 ± 2	235 ± 12
7k	84 ± 7	40 ± 3
7l	414 ± 44	173 ± 28
7m	410 ± 45	338 ± 10
7n	348 ± 20	93 ± 10
7o	446 ± 33	406 ± 15

[a] $[Ca^{2+}]_i$ was monitored for 20 min. Baseline $[Ca^{2+}]_i$ was 35 ± 4 nM. Results are expressed as the mean ± standard error of triplicate measurements. [b] NE = no significant elevation of $[Ca^{2+}]_i$ above baseline level.

The ability of the analogues to elevate $[Ca^{2+}]_i$ in TSU-Pr1 cells followed the relative SERCA inhibitory and apoptotic activities (Table 2).

In conclusion, a series of TG analogues containing an α-amino acid applicable for conjugation to PSA-specific peptides has been prepared. In general, the potency follows the relative lipophilicity of the analogues. The high activity of **7l** makes this analogue especially interesting as a drug moiety in a prodrug of TG.

Experimental Section

Chemistry. TG (**1**) was isolated from the seeds of *Thapsia garganica* L. as previously described.[1] Reagents and precursors were supplied by Aldrich and were used without further purification. Thin-layer chromatography was done using precoated aluminum sheets with Silica gel 60 F_{254} or RP-18 F_{254} (Merck). Compounds were visualized by inspection under UV (λ = 254 nm) or after spraying with naphthoresorcinol solution (0.2% w/v in ethanol diluted 1:1 with 2 M H_2SO_4) or ninhydrin solution (0.05% w/v in ethanol) followed by heating. Normal phase column chromatography (NPCC) was done with Silica gel 60, 40–63 μm (Merck) using mixtures of EtOAc–toluene–AcOH, 20:10:0.3 (A), heptane–acetone–AcOH, 7:3:0.1 (B), or toluene–acetone, 19:1 (C), and 1:1 (D). Reverse phase column chromatography (RPCC) was done with LiChroprep RP-18, 40–63 μm (Merck) using mixtures of MeOH-water, 4:1 (E), 5:1 (F), 6:1 (G), 7:1 (H), and 9:1 (I) or MeOH–water–AcOH, 9:1:0.1 (J). Melting points were measured with capillary tubes in an oil bath and were corrected. ^{1}H NMR (300 MHz) and ^{13}C NMR (75 MHz) spectra were recorded on a GEMINI 2000 BB, 300 MHz spectrometer. Chemical shift values (δ) are expressed in ppm relative to tetramethylsilane as internal standard. The following abbreviations are used for multiplicity of NMR signals: br = broad, s = singlet, d = doublet, t = triplet, q = quartet, dd = double doublet, m = multiplet. NMR signals corresponding to exchangeable protons are omitted. Signals from α-amino acids are assigned with Greek letters α, β, and γ. Signals originating from the TG nucleus of all the *O*-8 acylated compounds were in the following range: ^{1}H NMR ($CDCl_3$) δ 0.86–0.87 (t, J = 7–8 Hz, 3H, octanoyl H-8), 1.26–1.28 (m, 8H, octanoyl H-4 to H-7), 1.37–1.44 (br s, 3H, H-14), 1.39–1.50 (s, 3H, H-13), 1.58–1.60 (m, 2H, octanoyl H-3), 1.83–1.87 (br s, 3H, H-15), 1.88–1.90 (s, 3H, acetyl H-2), 1.90–1.92 (m, 3H, angeloyl C-2 CH_3), 1.99–2.00 (d, J = 7–8 Hz, 3H, angeloyl H-4), 2.28–2.30 (m, 2H, octanoyl H-2), 2.28–2.42 (dd, J = 3–4 and 13–15 Hz, 1H, H-9′), 2.89–3.03 (dd, J = 3–4 and 13–15 Hz, 1H, H-9), 4.18–4.33 (br s, 1H, H-1), 5.46–5.51 (m, 1H, H-2), 5.61–5.71 (m, 3H, H-3, H-6 and H-8), 6.10–6.12 (q, J = 7–8 Hz, 1H, angeloyl H-3). Some times H-8 appeared at a slightly lower ppm value than H-3 and H-6 and some times H-9′ was overlapped by signals from the acyl residues. ^{13}C NMR ($CDCl_3$) (labeled assignments are interchangeable) δ 12.8–13.4 (C-15), 14.1–14.4 (octanoyl C-8), 15.7–16.1 (C-13), 15.8–16.3 (angeloyl C-4), 19.6–20.8 (angeloyl C-2 CH_3), 22.2–22.8 (acetyl C-2), 22.5–22.8 (C-14), 23.6–24.4 (octanoyl C-7), 24.3–25.2 (octanoyl C-3), 29.0–29.5 (octanoyl C-4 to C-6), 34.6–36.1 (octanoyl C-2), 38.3–40.7 (C-9), 57.5–59.4 (C-1), 66.2–66.5 (C-8)[a], 77.0–77.8 (C-6)[a], 77.9–78.8 (C-2)[a], 78.5–78.9 (C-7)[b], 78.6–79.0 (C-11)[b], 84.3–85.7 (C-3)[a], 84.7–85.7 (C-10), 127.7–127.9 (angeloyl C-2), 130.1–131.2 (C-4)[c], 138.8–139.2 (angeloyl C-3), 141.1–142.1 (C-5)[c], 165.1–167.4 (C=O, angeloyl), 170.9–171.8 (C=O, acetyl), 172.9–173.1 (C=O, octanoyl), 174.5–177.6 (C=O, C-12). The identity of target compounds was confirmed with NMR and HRMS. The target compounds were pure according to TLC, and their NMR spectra showed no foreign signals other than minor solvent residuals.

8-*O*-Debutanoylthapsigargin (2). Triethylamine (2.5 mL) was added to a solution of **1** (0.80 mmol) in dry MeOH (50 mL) at room temperature. After 6 h at room temperature, the mixture was concentrated in vacuo. The residue was concentrated two times from toluene (50 mL) in vacuo to give **2** (yield 100%) as a white amorphous solid: ^{1}H NMR ($CDCl_3$) δ 0.87 (t, J = 7.0 Hz, 3H, octanoyl H-8), 1.28 (br s, 8H, octanoyl H-4 to H-7), 1.44 (s, 3H, H-14), 1.49 (s, 3H, H-13), 1.60 (m, 2H, octanoyl H-3), 1.84 (s, 3H, H-15), 1.90 (m, 6H, acetyl H-2 and angeloyl C-2 CH_3), 1.99 (d, J = 7.0 Hz, 3H, angeloyl H-4), 2.29 (m, 2H, octanoyl H-2), 2.47 (dd, J = 3.3 and 14.1 Hz, 1H, H-9′), 2.84 (d, J = 14.1 Hz, 1H, H-9), 3.57 (br s, 1H, H-8), 4.36 (s, 1H, H-1), 5.45 (m, 1H, H-2), 5.69 (s, 1H, H-3), 5.80 (s, 1H, H-6), 6.12 (q, J = 7.2 Hz, 1H, angeloyl H-3).

3-(4-Aminophenyl)propionic Acid Methyl Ester (8). Triethylamine (10.0 mmol) was added dropwise to a suspension of 4-amino-*trans*-cinnamic acid hydrochloride (10.0 mmol) in 2-methoxyethanol (12 mL) at room temperature. The suspension was filtered, and Pd-C 10% (120 mg) was added to the filtrate. The mixture was hydrogenated (4 atm) for 3 h at room temperature. The mixture was filtered through a column of Celite, and the filtrate was concentrated in vacuo to give 3-(4-aminophenyl)propionic acid (1.7 g) as a yellowish crystalline solid. This compound was esterified without further purification. Thionyl chloride (2.5 mL) was added dropwise to dry MeOH (10 mL) at −10 °C, and the solution was left for 10 min. 3-(4-Aminophenyl)propionic acid (1.7 g) was added to the solution, and the mixture was left overnight at room temperature. The solvent was removed in vacuo, and the residue was dissolved in EtOAc (200 mL). The solution was washed with 5% $NaHCO_3$ (200 mL), 10% NaCl (100 mL), and water (100 mL). The organic phase was dried ($MgSO_4$), filtered, and concentrated in vacuo to give **8** (yield 80%) as a crystalline yellow solid: ^{1}H NMR ($CDCl_3$) δ 2.55 (t, J = 7.0 Hz, 2H, H-2), 2.85 (t, J = 7.0 Hz, 2H, H-3), 3.65 (s, 3H, OCH_3), 6.60 (d, J = 9.0 Hz, 2H, Ar H-3 and H-5), 6.95 (d, J = 9.0 Hz, 2H, Ar H-2 and H-6).

3-(4-[N_α-*tert*-Butoxycarbonyl-L-leucinoylamino]phenyl)-propanoic Acid Methyl Ester (9). A solution of triphenylphosphine (1.00 mmol) in dry THF (1.0 mL) was under argon dropwise added to a solution of N_α-*tert*-butoxycarbonyl-L-leucine (1.00 mmol) and hexachloroacetone (0.50 mmol) in dry THF (2.0 mL) at −78 °C, and the solution was left for 30 min. A solution of compound **8** (1.00 mmol) in dry THF (1.0 mL)

and a solution of pyridine (6.00 mmol) in dry THF (6.0 mL) was added to the reaction mixture at −78 °C, and the mixture was left at room temperature for 1 h and filtered. The filtrate was concentrated in vacuo, and the residue was dissolved in CH_2Cl_2 (100 mL). The solution was washed twice with 1 M HCl (50 mL), twice with 5% $NaHCO_3$ (50 mL), and twice with 10% NaCl (50 mL) and concentrated in vacuo.P urification of the residue by NPCC (eluent A) afforded **9** (50%) as a yellowish crystalline solid: ^{1}H NMR ($CDCl_3$) δ 0.95 (m, 6H, Leu CH_3 and CH'_3), 1.43 (s, 9H, Boc CH_3), 1.62 (m, 1H, γ-H), 1.72 (m, 2H, β-H), 2.58 (t, J = 7.8 Hz, 2H, H-2), 2.88 (t, J = 7.8 Hz, 2H, H-3), 3.66 (s, 3H, OCH_3), 4.35 (m, 1H, α-H), 7.07 (d, J = 8.1 Hz, 2H, Ar H-2 and H-2′), 7.41 (d, J = 8.1 Hz, 2H, Ar H-3 and H-3′); ^{13}C NMR ($CDCl_3$) δ 22.3 (Leu CH_3), 23.6 (Leu CH'_3), 25.2 (γ-C), 28.5 (Boc CH_3), 31.0 (C-3), 36.5 (C-2), 41.7 (β-C), 52.1 (OCH_3), 54.5 (α-C), 81.2 (Boc *tert*-C), 120.3 (Ar C-3 and C-5), 129.0 (Ar C-2 and C-6), 132.7 (Ar C-1), 136.7 (Ar C-4), 156.4 (C=O, carbamate), 171.2 (C=O, C-1), 173.4 (C=O, amide).

3-(4-[N_α-*tert*-Butoxycarbonyl-L-leucinoylamino]phenyl)-propanoic Acid (10). 2 M NaOH (10 mL) was added at room temperature to a solution of **9** (4.89 mmol) in MeOH (65 mL), and the mixture was left for 10 min at room temperature. The MeOH was removed in vacuo, and the aqueous residue was acidified to pH 2 with 2 M H_2SO_4. The aqueous solution was extracted twice with EtOAc (60 mL), and the combined organic phases were washed with 10% w/v NaCl (60 mL) and water (60 mL). The organic phase was dried ($MgSO_4$), filtered, and concentrated in vacuo to give **10** (99%) as a yellowish crystalline solid: mp 64.5−66.5 °C; ^{1}H NMR ($CDCl_3$)δ 0.93 (d, J = 6.3 Hz, 3H, Leu CH_3), 0.96 (d, J = 6.3 Hz, 3H, Leu CH'_3), 1.38 (s, 9H, Boc CH_3), 1.66 (m, 1H, γ-H), 1.74 (m, 2H, β-H), 2.63 (t, J = 7.2 Hz, 2H, H-2), 2.89 (t, J = 7.2 Hz, 2H, H-3), 4.48 (m, 1H, α-H), 7.05 (d, J = 8.0 Hz, 2H, Ar H-2 and H-6), 7.43 (d, J = 8.0 Hz, 2H, Ar H-3 and H-5); ^{13}C NMR ($CDCl_3$) δ 21.9 (Leu CH_3), 23.0 (Leu CH'_3), 24.8 (γ-C), 28.4 (Boc CH_3), 30.2 (C-3), 35.8 (C-2), 41.4 (β-C), 54.0 (α-C), 80.5 (Boc *tert*-C), 120.4 (Ar C-3 and C-5), 128.9 (Ar C-2 and C-6), 136.4 (Ar C-1), 156.9 (C=O, carbamate), 176.5 (C=O, amide), 178.2 (C=O, acid); MS (FAB+) *m/z* 379 ([M+H]$^+$, 33%), 323 (100%).

8-*O*-(3-[4-{N_α-*tert*-Butoxycarbonyl-L-leucinoylamino}-phenyl]propanoyl)-8-*O*-debutanoylthapsigargin (11). To a mixture of **10** (0.26 mmol), **2** (0.26 mmol), and DMAP (0.03 mmol) in dry CH_2Cl_2(2.0 mL) was added a solution of DCC (0.29 mmol) in dry CH_2Cl_2 (0.75 mL) at 0 °C. The mixture was left for 1 h at 0 °C and then 7.5 h at room temperature. The mixture was filtered, and the filtrate was concentrated in vacuo. Purification of the residue by RPCC (eluent F) yielded **11** (50%) as a white amorphous solid: 3-(4-[N_α-*tert*-butoxycarbonyl-L-leucinoylamino]phenyl)propanoyl ^{1}H NMR ($CDCl_3$) δ 0.93 (d, J = 6.3 Hz, 3H, Leu CH_3), 0.97 (d, J = 6.3 Hz, 3H, Leu CH'_3) 1.40 (s, 9H, Boc CH_3), 1.62 (m, 1H, γ-H), 1.74 (m, 2H, β-H), 2.56 (t, J = 7.0 Hz, 2H, H-2), 2.83 (t, J = 7.0 Hz, 2H, H-3), 4.34 (m, 1H, α-H), 7.00 (d, J = 8.0 Hz, 2H, Ar H-2 and H-2′), 7.35 (d, J = 8.0 Hz, 2H, Ar H-3 and H-3′); ^{13}C NMR ($CDCl_3$) δ 23.0 (Leu CH'_3 and CH_3), 24.8 (γ-C), 28.4 (Boc CH_3), 29.8 (C-3), 36.2 (C-2), 40.9 (β-C), 53.9 (α-C), 80.6 (Boc *tert*-C), 120.5 (Ar C-3 and C-5), 128.7 (Ar C-2 and C-6), 136.3 (Ar C-4), 156.7 (C=O, carbamate), 172.0 (C=O, C-1), 176.5 (C=O, amide); HRMS (FAB+) *m/z* 963.4902 ([M+Na]$^+$, $C_{50}H_{73}N_2O_{15}Na$ requires 963.4830).

8-*O*-(3-[4-{L-Leucinoylamino}phenyl]propanoyl)-8-*O*-debutanoylthapsigargin (5). TFA (1.00 mL) was added to a solution of **11** (0.12 mmol) in dry CH_2Cl_2 (2.0 mL) at room temperature. The mixture was stirred for 1 h at room temperature and concentrated in vacuo to give **5** (yield 100%) as a yellowish amorphous solid: 3-(4-[L-leucinoylamino]phenyl)-propanoyl ^{1}H NMR ($CDCl_3$) δ 0.86 (m, 6H, Leu CH_3 and CH'_3), 1.59 (m, 1H, γ-H), 1.74 (m, 2H, β-H), 2.58 (br s, 2H, H-2), 2.83 (br s, 2H, H-3), 4.21 (m, 1H, α-H), 7.02 (br s, 2H, Ar H-2 and H-6), 7.22 (br s, 2H, Ar H-3 and H-5); ^{13}C NMR ($CDCl_3$) δ 23.0 (Leu CH'_3 and CH_3), 24.8 (γ-C), 29.8 (C-3), 36.2 (C-2), 40.9 (β-C), 53.9 (α-C), 120.5 (Ar C-3 and C-5), 128.7 (Ar C-2 and C-6), 136.3 (Ar C-4), 172.0 (C=O, C-1), 176.5 (C=O, amide); HRMS (FAB+) *m/z* 841.4551 ([M+H]$^+$, $C_{45}H_{65}N_2O_{13}$ requires 841.4487).

6-*tert*-Butoxycarbonylaminohexanoic Acid (12f). A solution of sodium hydroxide (1.47 mmol) in water (0.3 mL) was added to a solution of 6-aminohexanoic acid (1.50 mmol) in *tert*-butyl alcohol (3.0 mL), and the solution was left for 10 min at room temperature. Di-*tert*-butyl-dicarbonate (1.65 mmol) dissolved in *tert*-butyl alcohol (2.8 mL) was added to the solution, and the mixture was left overnight at room temperature. The mixture was concentrated in vacuo, and the residue was suspended in water (2.4 mL). The suspension was cooled on ice and acidified (pH 2) with 2 M H_2SO_4. The aqueous suspension was quickly extracted three times with EtOAc (3.6 mL), and the combined organic phases were washed three times with water (2.4 mL), dried ($MgSO_4$), filtered, and concentrated in vacuo. Purification of the residue by NPCC (eluent B) afforded **12f** (yield 61%) as white crystals: mp 38−39 °C; ^{1}H NMR ($CDCl_3$) δ 1.30 (m, 2H, H-4), 1.40 (s, 9H, Boc CH_3), 1.50 (m, 2H, H-3 or H-5), 1.57 (m, 2H, H-3 or H-5), 2.28 (t, J = 7.4 Hz, 2H, H-2), 3.01 (m, 2H, H-6).

Compounds **12g−j** were prepared as described for **12f** using 7-aminoheptanoic acid, 8-aminooctanoic acid, 11-aminoundecanoic acid, and 12-aminododecanoic acid, respectively, as starting materials.

7-*tert*-Butoxycarbonylaminoheptanoic Acid (12g). NPCC (eluent A) afforded **12g** (yield 82%) as white crystals: mp 55−56 °C; ^{1}H NMR ($CDCl_3$) δ 1.35 (m, 4H, H-4 and H-5), 1.45 (s, 9H, Boc CH_3), 1.48 (m, 2H, H-3 or H-6), 1.64 (m, 2H, H-3 or H-6), 2.35 (t, J = 7.5 Hz, 2H, H-2), 3.10 (m, 2H, H-7).

8-*tert*-Butoxycarbonylaminooctanoic Acid (12h). NPCC (eluent A) afforded **12h** (yield 79%) as white crystals: mp 56−57 °C; ^{1}H NMR ($CDCl_3$) δ 1.30 (m, 6H, H-4 to H-6), 1.45 (s, 9H, Boc CH_3), 1.46 (m, 2H, H-3 or H-7), 1.63 (m, 2H, H-3 or H-7), 2.34 (t, J = 7.4 Hz, 2H, H-2), 3.10 (m, 2H, H-8).

11-*tert*-Butoxycarbonylaminoundecanoic Acid (12i). NPCC (eluent A) afforded **12i** (yield 54%) as white crystals: mp 67−68 °C; ^{1}H NMR ($CDCl_3$) δ 1.28 (br s, 12H, H-4 to H-9), 1.45 (br s, 9H, Boc CH_3), 1.53 (m, 2H, H-3 or H-10), 1.63 (m, 2H, H-3 or H-10), 2.34 (t, J = 7.5 Hz, 2H, H-2), 3.10 (m, 2H, H-11).

12-*tert*-Butoxycarbonylaminododecanoic Acid (12j). NPCC (eluent B) afforded **12j** (yield 33%) as white crystals: mp 83.5−84.5 °C; ^{1}H NMR ($CDCl_3$) δ 1.27 (br s, 14H, H-4 to H-10), 1.45 (br s, 9H, Boc CH_3), 1.50 (m, 2H, H-3 or H-11), 1.63 (m, 2H, H-3 or H-11), 2.35 (t, J = 7.4 Hz, 2H, H-2), 3.10 (m, 2H, H-12).

8-*O*-(6-*tert*-Butoxycarbonylaminohexanoyl)-8-*O*-debutanoylthapsigargin (13f). Compound **2** (0.18 mmol), **12f** (0.20 mmol), and DMAP (0.20 mmol) was dissolved in dry CH_2Cl_2 (1.5 mL) at room temperature. After cooling on ice, a solution of DCC (0.20 mmol) in dry CH_2Cl_2 (0.5 mL) was added. The mixture was kept on ice for 1 h and then left for 5 h at room temperature. The mixture was filtered, and the filtrate was concentrated in vacuo. Purification of the residue by RPCC (eluent E) afforded **13f** (yield 54%) as a white amorphous solid: 6-*tert*-Butoxycarbonylaminohexanoyl ^{1}H NMR ($CDCl_3$) δ 1.28 (m, 2H, H-4), 1.43 (s, 9H, Boc CH_3), 1.61 (m, 4H, H-3 and H-5), 2.32 (m, 2H, H-2), 3.09 (m, 2H, H-6); ^{13}C NMR ($CDCl_3$) δ 25.2 (C-3), 25.8 (C-4), 28.7 (Boc CH_3), 29.5 (C-5), 34.8 (C-2), 40.7 (C-6), 80.1 (Boc *tert*-C), 172.7 (C=O, C-1); HRMS (FAB+) *m/z* 816.4139 ([M+Na]$^+$, $C_{41}H_{63}NO_{14}Na$ requires 816.4146).

Compounds **13g−j** were prepared as described for **13f**, using compounds **12g−j**, respectively, as starting materials.

8-*O*-(7-*tert*-Butoxycarbonylaminoheptanoyl)-8-*O*-debutanoylthapsigargin (13g). RPCC (eluent F) afforded **13g** (yield 41%) as a white amorphous solid: 7-*tert*-Butoxycarbonylaminoheptanoyl ^{1}H NMR ($CDCl_3$) δ 1.28 (m, 4H, H-4 to H-5), 1.44 (s, 9H, Boc CH_3), 1.60 (m, 4H, H-3 and H-6), 2.29 (m, 2H, H-2), 3.07 (m, 2H, H-7); ^{13}C NMR ($CDCl_3$) δ 25.0 (C-3), 28.6 (Boc CH_3), 29.0 (C-4 and C-5), 32.0 (C-6), 34.4 (C-2), 40.0 (C-7), 80.0 (Boc *tert*-C), 172.9 (C=O, C-1); HRMS (FAB+) *m/z* 830.4419 ([M+Na]$^+$, $C_{42}H_{65}NO_{14}Na$ requires 830.4303).

8-*O*-(8-*tert*-Butoxycarbonylaminooctanoyl)-8-*O*-debutanoylthapsigargin (13h). RPCC (eluent F) afforded **13h** (yield 52%) as a white amorphous solid: 8-*tert*-Butoxycarbonylaminooctanoyl ^{1}H NMR ($CDCl_3$) δ 1.30 (m, 6H, H-4 to H-6), 1.43 (s, 9H, Boc CH_3), 1.60 (m, 4H, H-3 and H-7), 2.29 (m, 2H, H-2), 3.07 (m, 2H, H-8); ^{13}C NMR ($CDCl_3$) δ 24.9 (C-3), 28.4 (Boc CH_3), 28.9 (C-4 to C-6), 31.6 (C-7), 34.3 (C-2), 40.3 (C-8), 79.8 (Boc *tert*-C), 172.9 (C=O, C-1); HRMS (FAB+) *m/z* 844.4340 ([M+Na]$^+$, $C_{43}H_{67}NO_{14}Na$ requires 844.4459).

8-*O*-(11-*tert*-Butoxycarbonylaminoundecanoyl)-8-*O*-debutanoylthapsigargin (13i). RPCC (eluent F) afforded **13i** (yield 64%) as a white amorphous solid: 11-*tert*-Butoxycarbonylaminoundecanoyl ^{1}H NMR ($CDCl_3$) δ 1.27 (m, 12H, H-4 to H-9), 1.44 (s, 9H, Boc CH_3), 1.60 (m, 4H, H-3 and H-10), 2.29 (m, 2H, H-2), 3.08 (m, 2H, H-11); ^{13}C NMR ($CDCl_3$) δ 25.0 (C-3), 28.6 (Boc CH_3), 29.1 (C-4 to C-8), 31.8 (C-10), 34.8 (C-2), 172.8 (C=O, C-1); HRMS (FAB+) *m/z* 886.5028 ([M+Na]$^+$, $C_{46}H_{73}NO_{14}Na$ requires 886.4929).

8-*O*-(12-*tert*-Butoxycarbonylaminododecanoyl)-8-*O*-debutanoylthapsigargin (13j). RPCC (eluent I) afforded **13j** (yield 77%) as a white amorphous solid: 12-*tert*-Butoxycarbonylaminododecanoyl ^{1}H NMR ($CDCl_3$)δ 1.26 (m, 14H, H-4 to H-10), 1.44 (s, 9H, Boc CH_3), 1.60 (m, 4H, H-3 and H-11), 2.28 (m, 2H, H-2), 3.09 (m, 2H, H-12); ^{13}C NMR ($CDCl_3$) δ 24.9 (C-3), 26.7 (C-10), 28.5 (Boc CH_3), 29.1 (C-4 to C-9), 31.7 (C-11), 34.6 (C-2), 41.0 (C-12), 172.8 (C=O, C-1); HRMS (FAB+) *m/z* 900.5084 ([M+Na]$^+$, $C_{47}H_{75}NO_{14}Na$ requires 900.5085).

8-*O*-(6-Aminohexanoyl)-8-*O*-debutanoylthapsigargin (6f). TFA (0.5 mL) was added to a solution of **13f** (0.05 mmol) in dry CH_2Cl_2 (3.0 mL) at room temperature. The mixture was left for 45 min at room temperature. Evaporation in vacuo afforded **6f** (yield 100%) as an amorphous yellowish solid: 6-Aminohexanoyl ^{1}H NMR ($CDCl_3$)δ 1.27 (m, 2H, H-4), 1.60 (m, 4H, H-3 and H-5), 2.31 (m, 2H, H-2), 2.97 (m, 2H, H-6); ^{13}C NMR ($CDCl_3$) δ 24.9 (C-3), 31.8 (C-5), 34.3 (C-2), 173.0 (C=O, C-1); HRMS (FAB+) *m/z* 694.3809 ([M+H]$^+$, $C_{36}H_{56}NO_{12}$ requires 694.3802).

Compounds **6g–j** were prepared as described for **6f**, using compounds **13g–j**, respectively, as starting materials.

8-*O*-(7-Aminoheptanoyl)-8-*O*-debutanoylthapsigargin (6g). Amorphous yelowish solid (yield 100%): 7-Aminoheptanoyl ^{1}H NMR ($CDCl_3$) δ 1.27 (m, 4H, H-4 to H-5), 1.60 (m, 4H, H-3 and H-6), 2.30 (m, 2H, H-2), 2.99 (m, 2H, H-7); ^{13}C NMR ($CDCl_3$) δ 25.0 (C-3), 29.0 (C-4 to C-5), 31.9 (C-6), 34.4 (C-2), 40.3 (C-7), 172.9 (C=O, C-1); HRMS (FAB+) *m/z* 708.3965 ([M+H]$^+$, $C_{37}H_{58}NO_{12}$ requires 708.3959).

8-*O*-(8-Aminooctanoyl)-8-*O*-debutanoylthapsigargin (6h). Amorphous yellowish solid (yield 100%): 8-Aminooctanoyl ^{1}H NMR ($CDCl_3$) δ 1.28 (m, 6H, H-4 to H-6), 1.60 (m, 4H, H-3 and H-7), 2.28 (m, 2H, H-2), 3.00 (m, 2H, H-8); ^{13}C NMR ($CDCl_3$)δ 24.9 (C-3), 29.0 (C-4 to C-6), 31.6 (C-7), 34.8 (C-2), 40.3 (C-8), 173.1 (C=O, C-1); HRMS (FAB+) *m/z* 722.4113 ([M+H]$^+$, $C_{38}H_{60}NO_{12}$ requires 722.4116).

8-*O*-(11-Aminoundecanoyl)-8-*O*-debutanoylthapsigargin (6i). Amorphous yellowish solid (yield 100%): 11-Aminoundecanoyl ^{1}H NMR ($CDCl_3$) δ 1.26 (m, 12H, H-4 to H-9), 1.59 (m, 4H, H-3 and H-10), 2.29 (m, 2H, H-2), 2.97 (m, 2H, H-11); ^{13}C NMR ($CDCl_3$)δ 24.9 (C-3), 28.8 (C-4 to C-9), 31.6 (C-10), 34.4 (C-2), 172.9 (C=O, C-1); HRMS (FAB+) *m/z* 764.4655 ([M+H]$^+$, $C_{41}H_{66}NO_{12}$ requires 764.4585).

8-*O*-(12-Aminododecanoyl)-8-*O*-debutanoylthapsigargin (6j). Amorphous yellowish solid (yield 100%): 12-Aminododecanoyl ^{1}H NMR ($CDCl_3$) δ 1.27 (m, 14H, H-4 to H-10), 1.60 (m, 4H, H-3 and H-11), 2.30 (m, 2H, H-2), 3.00 (m, 2H, H-12); ^{13}C NMR ($CDCl_3$) δ 24.9 (C-3), 29.1 (C-4 to C-10), 31.7 (C-11), 34.3 (C-2), 172.9 (C=O, C-1); HRMS (FAB+) *m/z* 778.4700 ([M+H]$^+$, $C_{42}H_{68}NO_{12}$ requires 778.4742).

6-Aminohexanoic Acid Methyl Ester Hydrochloride (14f). Thionyl chloride (4.0 mL) was slowly added to dry MeOH (30 mL) at −10 °C. After 10 min at −10 °C to the solution was added 6-aminohexanoic acid (15.25 mmol), and the mixture was left overnight at room temperature. The solution was concentrated in vacuo, and the residue was dissolved in MeOH (15 mL). To the solution was added Et_2O (60 mL) to precipitate the methyl ester hydrochloride. Filtration afforded **14f** (yield 84%) as white crystals: mp 118−122 °C; ^{1}H NMR (CD_3OD) δ 1.44 (m, 2H, H-4), 1.69 (m, 4H, H-3, and H-5), 2.37 (t, J = 7.5 Hz, 2H, H-2), 2.93 (t, J = 7.5 Hz, 2H, H-6), 3.66 (s, 3H, OCH_3); ^{13}C NMR (CD_3OD) δ 25.4 (C-3), 26.9 (C-4), 28.3 (C-2), 34.5 (C-5), 40.7 (C-6), 52.2 (OCH_3), 175.9 (C=O, C-1).

12-Aminododecanoic Acid Methyl Ester Hydrochloride (14j). Thionyl chloride (4.0 mL) was slowly added to dry MeOH (75 mL) at −10 °C. After 10 min at −10 °C, to the solution was added 12-aminododecanoic acid (13.93 mmol), and the mixture was left overnight at room temperature. The solution was concentrated in vacuo, and the residue was dissolved in MeOH (50 mL). To the solution was added Et_2O (80 mL) to precipitate the methyl ester hydrochloride. Filtration afforded **14j** (yield 93%) as white crystals: mp 160−161 °C; ^{1}H NMR (CD_3OD) δ 1.34 (m, 14H, H-4 to H-10), 1.62 (m, 4H, H-3 and H-11), 2.31 (t, J = 7.5 Hz, 2H, H-2), 2.91 (t, J = 7.5 Hz, 2H, H-12), 3.65 (s, 3H, OCH_3); ^{13}C NMR (CD_3OD) δ 26.1 (C-3), 27.5 (C-10), 28.6, 30.2, 30.3, 30.4, 30.5, 30.6, 30.6 (C-2 and C-4 to C-9), 34.9 (C-11), 40.9 (C-12), 52.1 (OCH_3), 176.3 (C=O, C-1).

6-(N_α-*tert*-Butoxycarbonyl-L-leucinoylamino)hexanoic Acid Methyl Ester (15k). N_α-*tert*-Butoxycarbonyl-L-leucine (5.50 mmol), **14f** (5.50 mmol), and DIPEA (5.50 mmol) was dissolved in dry CH_2Cl_2 (16.5 mL) at room temperature. To the mixture cooled on ice was added a solution of DCC (6.00 mmol) in dry CH_2Cl_2 (6.0 mL). After 3 h at room temperature, the mixture was filtered, and the filtrate was concentrated in vacuo. Purification of the residue by NPCC (eluent C) afforded **15k** (yield 50%) as a yellowish oil: ^{1}H NMR ($CDCl_3$) δ 0.93 (d, J = 6.5 Hz, 3H, Leu CH_3), 0.94 (d, J = 6.5 Hz, 3H, Leu CH'_3), 1.34 (m, 2H, H-4), 1.44 (s, 9H, Boc CH_3), 1.51 (m, 2H, H-5), 1.64 (m, 5H, H-3, β-H and γ-H), 2.31 (t, J = 7.5 Hz, 2H, H-2), 3.24 (m, 2H, H-6), 3.67 (s, 3H, OCH_3), 4.09 (m, 1H, α-H); ^{13}C NMR ($CDCl_3$) δ 22.1 (Leu CH_3), 23.0 (Leu CH'_3), 24.5 (γ-C), 24.8 (C-3), 26.3 (C-4), 28.4 (Boc CH_3), 29.2 (C-5), 33.9 (C-2), 39.2 (β-C), 41.4 (C-6), 51.6 (OCH_3), 53.2 (α-C), 80.0 (Boc *tert*-C), 156.1 (C=O, carbamate), 173.0 (C=O, C-1), 174.3 (C=O, amide); HRMS (FAB+) *m/z* 359.2522 ([M+H]$^+$, $C_{18}H_{35}N_2O_5$ requires 359.2546).

Compounds **15l,m** were prepared as described for **15k**, using N_α-*tert*-butoxycarbonyl-L-leucine and N_α-*tert*-butoxycarbonyl-L-alanine, respectively, together with compound **14j** as starting materials.

12-(N_α-*tert*-Butoxycarbonyl-L-leucinoylamino)dodecanoic Acid Methyl Ester (15l). NPCC (eluent C) afforded **15l** (yield 53%) as white crystals: mp 63−64 °C; ^{1}H NMR ($CDCl_3$) δ 0.93 (m, 6H, Leu CH_3 and CH'_3), 1.26 (br s, 14H, H-4 to H-10), 1.44 (s, 9H, Boc CH_3), 1.48 (m, 3H, β-H and γ-H), 1.63 (m, 4H, H-3 and H-11), 2.31 (t, J = 7.5 Hz, 2H, H-2), 3.23 (m, 2H, H-12), 3.67 (s, 3H, OCH_3), 4.08 (m, 1H, α-H); ^{13}C NMR ($CDCl_3$) δ 22.2 (Leu CH_3), 22.9 (Leu CH'_3), 24.8 (γ-C), 25.0 (C-3), 26.9 (C-10), 28.4 (Boc CH_3), 29.2, 29.3, 29.5 (C-4 to C-9 and C-11), 34.2 (C-2), 39.5 (β-C), 41.4 (C-12), 51.6 (OCH_3), 53.2 (α-C), 80.1 (Boc *tert*-C), 156.1 (C=O, carbamate), 172.8 (C=O, C-1), 174.7 (C=O, amide); HRMS (FAB+) *m/z* 443.3517 ([M+H]$^+$, $C_{24}H_{47}N_2O_5$ requires 443.3485).

12-(N_α-*tert*-Butoxycarbonyl-L-alaninoylamino)dodecanoic Acid Methyl Ester (15m). NPCC (eluent C) afforded **15m** (yield 55%) as white crystals: ^{1}H NMR (CD_3OD) δ 1.30 (br s, 14H, H-4 to H-10), 1.44 (s, 9H, Boc CH_3), 1.49 (m, 3H, Ala CH_3), 1.59 (m, 4H, H-3 and H-11), 2.31 (t, J = 7.5 Hz, 2H, H-2), 3.17 (m, 2H, H-12), 3.65 (s, 3H, OCH_3), 3.99 (m, 1H, α-H); ^{13}C NMR (CD_3OD) δ 18.6 (β-C), 26.1 (C-3), 28.0 (C-10), 28.8 (Boc CH_3), 30.3, 30.5, 30.7 (C-4 to C-9 and C-11), 34.9 (C-2), 40.4 (C-12), 51.8 (OCH_3), 52.1 (α-C), 80.7 (Boc *tert*-C), 158.2 (C=O, carbamate), 176.3 (C=O, amide); HRMS (FAB+) *m/z* 401.3036 ([M+H]$^+$, $C_{21}H_{41}N_2O_5$ requires 401.3015).

6-(N_α-*tert*-Butoxycarbonyl-L-leucinoylamino)hexanoic Acid (16k). 2 M NaOH (10 mL) was added to a solution of **15k** (0.5 mmol) in MeOH (20 mL), and the mixture was left for 40 min at room temperature. The MeOH was removed in vacuo, and the aqueous residue was cooled on ice and acidified to pH 2 with 2 M H_2SO_4. The aqueous solution was extracted

three times with EtOAc (50 mL) and the combined organic phases were washed with 10% w/v NaCl (25 mL) and water (25 mL). The organic phase was dried ($MgSO_4$) and filtered. Concentration in vacuo afforded **16k** (yield 90%) as white crystals: mp 100.5−102.5 °C; ^{1}H NMR ($CDCl_3$) δ 0.91 (d, J = 4.5 Hz, 3H, Leu CH_3), 0.93 (d, J = 4.5 Hz, 3H, Leu CH'_3), 1.37 (m, 2H, H-4), 1.43 (s, 9H, Boc CH_3), 1.51 (m, 3H, β-H and γ-H), 1.65 (m, 4H, H-3 and H-5), 2.34 (t, J = 7.5 Hz, 2H, H-2), 3.24 (m, 2H, H-6), 4.15 (m, 1H, α-H); ^{13}C NMR ($CDCl_3$) δ 22.1 (Leu CH_3), 22.8 (Leu CH'_3), 24.4 (γ-C), 24.8 (C-3), 26.2 (C-4), 28.4 (Boc CH_3), 29.0 (C-5), 33.9 (C-2), 39.3 (β-C), 41.3 (C-6), 53.2 (α-C), 80.3 (Boc *tert*-C), 156.4 (C=O, carbamate), 173.3 (C=O, amide), 177.9 (C=O, C-1); HRMS (FAB+) *m/z* 345.2430 ($[M+H]^+$, $C_{17}H_{33}N_2O_5$ requires 345.2389).

Compounds **16l,m** were prepared as described for **16k**, using compounds **15l,m**, respectively, as starting materials.

12-(N_α-*tert*-Butoxycarbonyl-L-leucinoylamino)dodecanoic Acid (16l). Yellowish oil (yield 95%): ^{1}H NMR ($CDCl_3$) δ 0.93 (m, 6H, Leu CH_3 and CH'_3), 1.27 (br s, 14H, H-4 to H-10), 1.43 (s, 9H, Boc CH_3), 1.48 (m, 3H, β-H and γ-H), 1.62 (m, 4H, H-3 and H-11), 2.35 (t, J = 7.5 Hz, 2H, H-2), 3.23 (m, 2H, H-12), 4.13 (br s, 1H, α-H); ^{13}C NMR ($CDCl_3$) δ 22.1 (Leu CH_3), 22.8 (Leu CH'_3), 24.7 (γ-C), 24.8 (C-3), 26.6 (C-10), 28.3 (Boc CH_3), 28.7, 28.9, 29.1, 29.3 (C-4 to C-9 and C-11), 34.0 (C-2), 39.5 (β-C), 41.3 (C-12), 53.1 (α-C), 80.3 (Boc *tert*-C), 156.4 (C=O, carbamate), 173.1 (C=O, amide), 178.3 (C=O, C-1); HRMS (FAB+) *m/z* 429.3356 ($[M+H]^+$, $C_{23}H_{45}N_2O_5$ requires 429.3328).

12-(N_α-*tert*-Butoxycarbonyl-L-alaninoylamino)dodecanoic Acid (16m). Amorphous solid (yield 93%): ^{1}H NMR (CD_3OD) δ 1.30 (br s, 14H, H-4 to H-10), 1.44 (s, 9H, Boc CH_3), 1.49 (m, 3H, Ala CH_3), 1.59 (m, 4H, H-3 and H-11), 2.27 (t, J = 7.5 Hz, 2H, H-2), 3.18 (m, 2H, H-12), 4.00 (m, 1H, α-H); ^{13}C NMR (CD_3OD) δ 18.8 (β-C), 26.3 (C-3), 28.2 (C-10), 28.9 (Boc CH_3), 30.7, 30.9, 31.1 (C-4 to C-9 and C-11), 35.2 (C-2), 40.6 (C-12), 52.0 (α-C), 80.8 (Boc *tert*-C), 175.8 (C=O, amide), 177.8 (C=O, C-1); HRMS (FAB+) *m/z* 387.2807 ($[M+H]^+$, $C_{20}H_{39}N_2O_5$ requires 387.2859).

8-*O*-(6-[N_α-*tert*-Butoxycarbonyl-L-leucinoylamino]hexanoyl)-8-*O*-debutanoylthapsigargin (17k). Compound **2** (0.36 mmol), **16k** (0.36 mmol), and DMAP (0.04 mmol) was dissolved in dry CH_2Cl_2 (2.0 mL) at room temperature. To the mixture cooled on ice was added a solution of DCC (0.40 mmol) in dry CH_2Cl_2 (1.0 mL). The mixture was left on ice for 1 h and then left for 3.5 h at room temperature. The mixture was filtered, and the filtrate was concentrated in vacuo. Purification of the residue by RPCC (eluent E) afforded **17k** (yield 69%) as a white amorphous solid: 6-(N_α-*tert*-Butoxycarbonyl-L-leucinoylamino)hexanoyl ^{1}H NMR ($CDCl_3$) δ 0.92 (m, 6H, Leu CH_3 and CH'_3), 1.28 (br s, 2H, H-4), 1.42 (br s, 9H, Boc CH_3), 1.61 (m, 4H, H-3 and H-5), 2.30 (m, 2H, H-2), 3.20 (m, 2H, H-6), 4.06 (m, 1H, α-H); ^{13}C NMR ($CDCl_3$) δ 22.6 (Leu CH_3 and CH'_3), 24.8 (γ-C), 24.9 (C-3), 28.4 (Boc CH_3), 29.1 (C-4), 31.7 (C-5), 34.3 (C-2), 38.4 (β-C), 41.2 (C-6), 53.1 (α-C), 80.1 (Boc *tert*-C) 156.3 (C=O, carbamate), 172.9 (C=O, C-1), 173.6 (C=O, amide); HRMS (FAB+) *m/z* 907.5177 ($[M+H]^+$, $C_{47}H_{75}N_2O_{15}$ requires 907.5167).

Compounds **17l,m** were prepared as described for **17k**, using compounds **16l,m**, respectively, as starting materials.

8-*O*-(12-[N_α-*tert*-Butoxycarbonyl-L-leucinoylamino]dodecanoyl)-8-*O*-debutanoylthapsigargin (17l). RPCC (eluent D) afforded **17l** (yield 94%) as a white amorphous solid: 12-(N_α-*tert*-Butoxycarbonyl-L-leucinoylamino)dodecanoyl ^{1}H NMR ($CDCl_3$) δ 0.92 (m, 6H, Leu CH_3 and CH'_3), 1.26 (br s, 14H, H-4 to H-10), 1.42 (br s, 9H, Boc CH_3), 1.61 (m, 4H, H-3 and H-11), 2.28 (m, 3H, H-2), 3.20 (m, 2H, H-12), 4.05 (m, 1H, α-H); ^{13}C NMR ($CDCl_3$) δ 22.6 (Leu CH_3 and CH'_3), 24.8 (γ-C), 24.9 (C-3), 26.7 (C-10), 28.4 (Boc CH_3), 29.0−29.3 (C-4 to C-9), 31.7 (C-11), 34.3 (C-2), 38.4 (β-C), 41.1 (C-12), 53.1 (α-C), 80.1 (Boc *tert*-C), 156.2 (C=O, carbamate), 172.9 (C=O, C-1), 173.0 (C=O, amide); HRMS (FAB+) *m/z* 1013.5938 ($[M+Na]^+$, $C_{53}H_{86}N_2O_{15}Na$ requires 1013.5926).

8-*O*-(12-[N_α-*tert*-Butoxycarbonyl-L-alaninoylamino]dodecanoyl)-8-*O*-debutanoylthapsigargin (17m). RPCC (eluent F) afforded **17m** (yield 78%) as a white amorphous solid: 12-(N_α-*tert*-Butoxycarbonyl-L-alaninoylamino)dodecanoyl ^{1}H NMR ($CDCl_3$) δ 1.26 (br s, 14H, H-4 to H-10), 1.43 (s, 9H, Boc CH_3), 1.47 (m, 3H, Ala CH_3), 1.60 (m, 4H, H-3 and H-11), 2.28 (m, 2H, H-2), 3.22 (m, 2H, H-12), 4.12 (m, 1H, α-H); ^{13}C NMR ($CDCl_3$) δ 18.2 (β-C), 24.9 (C-3), 26.6 (C-10), 28.3 (Boc CH_3), 28.8, 29.1, 29.5 (C-4 to C-9), 31.7 (C-11), 34.5 (C-2), 50.0 (α-C), 80.6 (Boc *tert*-C), 173.0 (C=O, C-1 and amide); HRMS (FAB+) *m/z* 949.5622 ($[M+H]^+$, $C_{50}H_{81}N_2O_{15}$ requires 949.5637).

8-*O*-(6-[L-Leucinoylamino]hexanoyl)-8-*O*-debutanoylthapsigargin (7k). TFA (1.2 mL) was added to a solution of **17k** (0.20 mmol) in dry CH_2Cl_2 (3.0 mL) at room temperature. The mixture was left for 45 min at room temperature. Evaporation in vacuo afforded **7k** (yield 100%) as an amorphous yellowish solid: 6-(L-Leucinoylamino)hexanoyl ^{1}H NMR ($CDCl_3$) δ 0.93 (m, 6H, Leu CH_3 and CH'_3), 1.28 (m, 2H, H-4), 1.60 (m, 4H, H-3 and H-5), 2.29 (m, 2H, H-2), 3.20 (m, 2H, H-6), 3.62 (m, 1H, α-H); ^{13}C NMR ($CDCl_3$) δ 22.6 (Leu CH_3 and CH'_3), 24.8 (γ-C), 24.9 (C-3), 29.0 (C-4), 31.7 (C-5), 34.3 (C-2), 38.3 (β-C), 44.3 (C-6), 53.6 (α-C), 170.8 (C=O, C-1), 172.9 (C=O, amide); HRMS (FAB+) *m/z* 807.4624 ($[M+H]^+$, $C_{42}H_{67}N_2O_{13}$ requires 807.4643).

Compounds **7l,m** were prepared as described for **7k**, using compounds **17l,m**, respectively, as starting materials.

8-*O*-(12-[L-Leucinoylamino]dodecanoyl)-8-*O*-debutanoylthapsigargin (7l). Amorphous yellowish solid (yield 100%): 12-(L-Leucinoylamino)dodecanoyl ^{1}H NMR ($CDCl_3$) δ 0.95 (m, 6H, Leu CH_3 and CH'_3), 1.25 (br s, 14H, H-4 to H-10), 1.61 (m, 4H, H-3 and H-11), 2.33 (m, 2H, H-2), 3.25 (m, 2H, H-12), 4.19 (m, 1H, α-H); ^{13}C NMR ($CDCl_3$) δ 22.6 (Leu CH_3 and CH'_3), 24.6 (C-3), 24.8 (γ-C), 26.5 (C-10), 28.8−29.1 (C-4 to C-9), 31.7 (C-11), 34.4 (C-2), 38.1 (β-C), 40.5 (C-12), 53.3 (α-C), 173.1 (C=O, C-1), 174.5 (C=O, amide); HRMS (FAB+) *m/z* 891.5641 ($[M+H]^+$, $C_{48}H_{79}N_2O_{13}$ requires 891.5582).

8-*O*-(12-[L-Alaninoylamino]dodecanoyl)-8-*O*-debutanoylthapsigargin (7m). Amorphous yellowish solid (yield 100%): 12-(L-Alaninoylamino)dodecanoyl ^{1}H NMR ($CDCl_3$) δ 1.24 (m, 14H, H-4 to H-10), 1.53 (m, 3H, Ala CH_3), 1.57 (m, 4H, H-3 and H-11), 2.30 (m, 2H, H-2), 3.20 (br s, 2H, H-12), 4.22 (br s, 1H, α-H); ^{13}C NMR ($CDCl_3$) δ 17.4 (β-C), 24.9 (C-3), 26.8 (C-10), 28.8, 29.3, 29.6 (C-4 to C-9), 31.8 (C-11), 34.6 (C-2), 50.4 (α-C), 174.1 (C=O, amide); HRMS (FAB+) *m/z* 849.5057 ($[M+H]^+$, $C_{45}H_{73}N_2O_{13}$ requires 849.5112).

8-*O*-(12-[N_α-*tert*-Butoxycarbonyl-L-serinoylamino]dodecanoyl)-8-*O*-debutanoylthapsigargin (18n). *N*-*tert*-Butoxycarbonyl-L-serine (0.18 mmol), **6j** (0.18 mmol), and HOBT (0.18 mmol) were dissolved in dry DMF (2.0 mL) at room temperature. To the mixture cooled on ice was added a solution of DCC (0.18 mmol) in dry DMF (1.0 mL). The mixture was left on ice for 1 h and then left for 3.5 h at room temperature. The mixture was filtered, and the filtrate was concentrated in vacuo. Purification of the residue by RPCC (eluent J) afforded **18n** (yield 72%) as a white amorphous solid: 12-(N_α-*tert*-Butoxycarbonyl-L-serinoylamino)dodecanoyl ^{1}H NMR ($CDCl_3$) δ 1.27 (br s, 14H, H-4 to H-10), 1.45 (s, 9H, Boc CH_3), 1.60 (m, 4H, H-3 and H-11), 2.29 (m, 2H, H-2), 3.24 (t, J = 6.2 Hz, 2H, H-12), 3.64 (m, 1H, β-H'), 4.05 (dd, J = 3.0 and 11.1 Hz, 1H, β-H), 4.11 (m, 1H, α-H); ^{13}C NMR ($CDCl_3$) 24.9 (C-3), 26.6 (C-10), 28.3 (Boc CH_3), 28.8−29.3 (C-4 to C-9), 31.7 (C-11), 34.3 (C-2), 39.6 (C-12), 62.8 (β-C), 80.8 (Boc *tert*-C), 156.6 (C=O, carbamate), 171.6 (C=O, C-1), 173.2 (C=O, amide); HRMS (FAB+) *m/z* 965.5593 ($[M+H]^+$, $C_{50}H_{81}N_2O_{16}$ requires 965.5586).

Compound **18o** was prepared as described for **18n**, using N_α-*tert*-butoxycarbonyl-L-phenylalanine as starting material.

8-*O*-(12-[N_α-*tert*-Butoxycarbonyl-L-phenylalaninoylamino]dodecanoyl)-8-*O*-debutanoylthapsigargin (18o). RPCC (eluent J) afforded **18o** (yield 73%) as a white amorphous solid: 12-(N_α-*tert*-Butoxycarbonyl-L-phenylalaninoylamino)dodecanoyl ^{1}H NMR ($CDCl_3$)δ 1.26 (br s, 14H, H-4 to H-10), 1.38 (br s, 9H, Boc CH_3), 1.58 (m, 6H, H-3 and H-11), 2.28 (m, 3H, H-2), 3.01 (m, 2H, β-H), 3.13 (m, 2H, H-12), 4.25 (m, 1H, α-H), 7.18−7.29 (m, 5H, Ph); ^{13}C NMR ($CDCl_3$) δ 24.9 (C-3), 26.7 (C-10), 28.3 (Boc CH_3), 29.0−29.3 (C-4 to C-9), 31.7

(C-11), 34.3 (C-2), 38.4 (β-C), 41.1 (C-12), 62.0 (α-C), 81.4 (Boc *tert*-C), 127.0 (Phe C-4), 128.8 (Phe C-2, C-2′), 129.5 (Phe C-3, C-3′), 137.0 (Phe C-1), 158.3 (C=O, carbamate), 172.9 (C=O, C-1); HRMS (FAB+) *m/z* 1025.606 ($[M+H]^+$, $C_{56}H_{85}N_2O_{15}$ requires 1025.595).

Compounds **7n,o** were prepared as described for **7k**, using compounds **18n,o**, respectively, as starting materials.

8-*O*-(12-[L-Serinoylamino]dodecanoyl)-8-*O*-debutanoylthapsigargin (7n). Amorphous yellowish solid (yield 100%): 12-(L-Serinoylamino)dodecanoyl ^{1}H NMR ($CDCl_3$) δ 1.26 (br s, 14H, H-4 to H-10), 1.58 (m, 4H, H-3 and H-11), 2.29 (m, 2H, H-2), 3.17 (br s, 2H, H-12), 3.74 (dd, J = 13.7 and 6.8 Hz, 1H, β-H′), 3.88 (br s, 1H, α-H), 4.01 (br s, 1H, β-H); ^{13}C NMR ($CDCl_3$) δ 24.9 (C-3), 26.6 (C-10), 28.8–29.1 (C-4 to C-9), 31.7 (C-11), 34.3 (C-2), 55.4 (α-C), 59.6 (β-C), 173.0 (C=O, C-1), 174.4 (C=O, amide); HRMS (FAB+) *m/z* 865.5010 ($[M+H]^+$, $C_{45}H_{73}N_2O_{14}$ requires 865.5062).

8-*O*-(12-[L-Phenylalaninoylamino]dodecanoyl)-8-*O*-debutanoylthapsigargin (7o). Amorphous yellowish solid (yield 100%): 12-(L-Phenylalaninoylamino)dodecanoyl ^{1}H NMR ($CDCl_3$) δ 1.27 (m, 14H, H-4 to H-10), 1.60 (m, 6H, H-3 and H-11), 2.28 (m, 2H, H-2), 2.68 (dd, J = 9.3 and 13.7 Hz, 1H, β-H), 3.23 (m, 3H, β-H′ and H-12), 3.58 (dd, J = 9.3 and 4.2 Hz, 1H, α-H), 7.20–7.34 (m, 5H, Ph); ^{13}C NMR ($CDCl_3$) δ 24.9 (C-3), 26.7 (C-10), 29.0–29.3 (C-4 to C-9), 31.7 (C-11), 34.3 (C-2), 38.4 (β-C), 41.1 (C-12), 61.1 (α-C), 127.0 (Phe C-4), 128.9 (Phe C-2, C-2′), 129.5 (Phe C-3, C-3′), 138.1 (Phe C-1), 172.9 (C=O, C-1); HRMS (FAB+) *m/z* 925.539 ($[M+H]^+$, $C_{51}H_{77}N_2O_{13}$ requires 925.543).

Isolation of Sarcoplasmic Reticulum (SR). Frozen rabbit muscle was purchased from Pel-Freez Biologicals (Rogers, AR) and MOPS, sucrose, EDTA, and KCl were purchased from SIGMA. Homogenization was done with a commercial blender (Waring, USA). Centrifugation was done with a Sorvall RC-5B superspeed centrifuge (DuPont, USA) and a L7 ultracentrifuge (Beckman Coulter, USA). The temperature was kept at 0–4 °C during the preparation. Frozen rabbit muscle (180 g) was blended 15 s every 5 min during 1 h with 510 mL of a solution containing 10 mM MOPS, pH 7.0, 10% sucrose and 0.1 mM EDTA. The pH was kept between 6.5 and 7.0 by adding 10% NaOH. The homogenate was centrifuged at 15000*g* for 20 min. The supernatant was filtered through a path of cheesecloth, and centrifuged at 40000*g* for 90 min. The pellet was suspended with a Dounce glass homogenizer in 60 mL of a solution containing 10 mM MOPS, pH 7.0, and 0.6 M KCl. After incubating for 40 min at 4 °C, the suspension was centrifuged at 15000*g* for 20 min. The 10% top of the supernatant and the pellet were discarded. The supernatant was collected and centrifuged at 40000*g* for 90 min. The pellet was suspended with a Dounce glass homogenizer in 40 mL of microsome storage solution containing 10 mM MOPS, pH 7.0 and 30% sucrose. The microsomes were stored at −80 °C. The sarcoplasmic reticulum (SR) protein concentration (1.1 mg/mL) was determined with the Micro BCA Protein Assay Reagent kit supplied by Pierce (Rockford, IL) using bovine serum albumin (BSA) as standard.

Measurement of ATPase Activity. KCl, Trizma-HCl, $MgCl_2$, EGTA, $CaCl_2$, β-NADH, phosphoenolpyruvate (PEP), A23187, phosphoenolpyruvate kinase (PK), lactate dehydrogenase (LDH), and ATP were supplied by Sigma. The ATPase activity was measured with a Spectramax Plus^{384} microplate spectrophotometer (Molecular Devices Corporation, Sunnyvale, CA) as the rate of ATP hydrolysis essentially as previously described.[66,128] Buffer A: 0.1 M KCl, 20 mM Trizma-HCl, pH 7.5, 5 mM $MgCl_2$, 0.5 mM EGTA, 0.7 mM $CaCl_2$. Solution 1: 1.2 mM β-NADH, 1.5 mM PEP, 4.5 μM A23187, 22.5 U/mL PK, 54 U/mL LDH and 30 μg/mL SR protein in buffer A. Solution 2: Control or inhibitor dilutions in buffer A (concentrations corrected for a 1:3 dilution). Solution 3: 0.72 mM ATP in buffer A. 100 μL of solution 1 was mixed with 100 μL of solution 2 and 100 μL of solution 3 was added to start the reaction. After 5 min of incubation, the OD_{340} was measured kinetically at room temperature (n = 3) for at least 10 min. Typically, a 1 mM DMSO solution of inhibitor was diluted 1:100 in buffer A before making serial dilutions in buffer A. The amount of DMSO present did not influence the measured ATPase activity. The total ATPase activity was 7.0 μmol of ATP (mg of SR protein)$^{-1}$ min^{-1}.

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